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PATENT- OG VAREMÆRKESTYRELSEN

LIPID-BASED DRUG DELIVERY SYSTEMS CONTAINING PHOSPHOLIPASE A2 DEGRADABLE LIPID DERIVATIVES AND THE THERAPEUTIC USES THEREOF

FIELD OF THE INVENTION

The invention relates to lipid-based pharmaceutical compositions used in the treatment of various disorders, e.g. cancer, infectious, and inflammatory conditions, psoriasis, etc., i.e. disorders and diseases associated with or resulting from increased levels of PLA_2 activity in the diseased tissue.

BACKGROUND OF THE INVENTION

Mono-ether lyso-phospholipids and alkyl phosphocholines are known to be effective anticancer agents (see e.g. US 3,752,886 and later references). Several mechanisms of the toxic action of ether-lipids towards cancer cells have been proposed involving lack of alkyl-cleavage enzymes in cancer cells. This leads to accumulation of the ether-lipids in the cell membranes which induce membrane defects an possibly subsequent lysis. Other potential mechanisms of action include effects on intracellular protein phosphorylation and disruption of the lipid metabolism. Normal cells typically possess alkyl-cleavage enzymes, which enable them to avoid the toxic effect of ether-lipids. However, some normal cells e.g., red blood cells, have like cancer cells no means of avoiding the disruptive effect of the etherlipids. Accordingly, therapeutic use of ether-lipids requires an effective drug-delivery system that protects the normal cells from the toxic effects.

Lohmeyer and Workman, Brit. J. Cancer, describe the cytotoxic effect of arachidonoyl-PAF_{16} in vitro. However, the reference does not describe or suggest the mode of action for this compound.

BRIEF DESCRIPTION OF THE INVENTION

The new principle for liposomal drug targeting, release and absorption by endogeneous PLA_2 described and documented in this paper within a biophysical drug-delivery model system can be further generalised to a case also involving lipid-based prodrugs as illustrated in Fig. 9. In this case a specific lipid-analogue compound may be incorporated into the carrier liposome and act as a prodrug which is turned into an active drug by

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It is hereby certified:

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hydrolysis via the endog neous phospholipase. A possible example could be certain mono-ether lipids which have been found to xhibit anti-cancer activity. If the mono-ether lipids are modified with a long fatty-acid chain that is ester linked in the sn-2-position and therefore can be hydrolysed by PLA_2 at the target site, these modified mono-ether lipids constituting the carrier liposome will act as prodrugs. Finally it should be pointed out that certain drugs such as the anti-cancer drug adriamycin (of which doxorubicin is a derivative) themselves are known to stimulate PLA_2-activity by decreasing the calcium requirement of the enzyme.

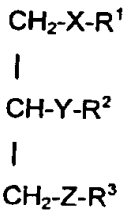
The principle of drug targeting, release and absorption by endogeneous phospholipase A2 (PLA_2) which is illustrated in Fig. 8, can be applied to a case also involving lipid-based prodrugs. In this case lipid derivatives are constituents of the carrier liposome and act as prodrugs which are turned into active drugs (e.g. ether lipids) by hydrolysis via the endogeneous PLA_2 that is present in elevated concentrations in the diseased target tissue. A specific example is a prodrug of a certain mono-ether lipid which exhibits anti-cancer activity. A therapeutically active compound (e.g. regulatory fatty acid derivatives) that is ester bound to the phospholipid in the sn-2 position and therefore renders the lipid derivative substrate for PLA_2. If the mono-ether lipids are modified with, e.g. a ester-linked derivative in the sn-2-position and therefore can be hydrolysed by PLA_2 at the target site, these lipid derivatives constituting the carrier liposome will act as prodrugs that become hydrolysed and turned into drugs by PLA_2 at the target site. In this way therapeutically active substances, e.g., monoether lipids and ester-linked derivatives will be liberated at the desired target site. Pharmaceutical compositions containing the lipid-based system can be used therapeutically, for example, in the treatment of cancer, infectious and inflammatory conditions.

Also included is the prophylactic use, the use as antiviral agent, use against leishmania parasite and also against multiple sclerosis.

This invention provides such a delivery system in the form of lipid-based carriers, e.g. liposomes, composed of lipid-bilayer forming ether-lipids such as glycerophospholipids containing an alkyl-linkage in the 1-position and an acyl-linkage in the sn-2-position on the glycerol backbone. In addition, the carrier system may contain lipid-bilayer stabilising components, e.g. lipopolymers and sterols which lead to an increased vascular circulation time and as a consequence an accumulation in the diseased target tissue. When the

carriers reach the target site of therapeutic action, e.g. cancer cells, PLA_2-catalyzed hydrolysis of the acyl-linkag releases the therapeutically active components, typically lyso-etherlipids and ester-linked derivatives. Contradictory to alkyl-cleavage enzymes which are nearly absent in cancer cells, PLA_2 activity is elevated in cancer tissue. In addition, PLA_2 activity is elevated in diseased regions such as inflammatory tissue.

Thus, the present invention i.a. relates to lipid derivatives of the following formula:



wherein

X and Z independently are selected from O, CH_2, NH, NMe, S, S(O), and $S(O)_2$, preferably from O, NH, NMe and CH_2, in particular O and CH_2;

Y is -OC(O)-, Y then being connected to R^2 via either the oxygen or carbonyl carbon atom, preferably via the carbonyl carbon atom, or Y is -NHC(O)-, Y then being connected to R^2 via either the nitrogen or carbonyl carbon atom, preferably via the carbonyl carbon atom;

R^1 is an aliphatic group of the formula Y^1Y^2;

R^2 is an aliphatic group having a length of at least 7, preferably at least 9, carbon atoms, preferably a group of the formula Y^1Y^2;

where Y^1 is $-(CH_2)_{n1}-(CH=CH)_{n2}-(CH_2)_{n3}-(CH=CH)_{n4}-(CH_2)_{n5}-(CH=CH)_{n6}-(CH_2)_{n7}-(CH=CH)_{n8r}-(CH_2)_{n9}$, and the sum of n1+2n2+n3+2n4+n5+2n6+n7+2n8+n9 is an integer of from 9 to 29; n1 is zero or an integer of from 1 to 29, n3 is zero or an integer of from 1 to 20, n5 is zero or an integer of from 1 to 17, n7 is zero or an integer of from 1 to 14, and n9 is zero or an integer of from 1 to 11; and each of n2, n4, n6 and n8 is independently zero or 1; and Y^2 is CH_3 or CO_2H; where each Y^1-Y^2 independently may be substituted with halogen or C_{1-4}-alkyl, but preferably Y^1-Y^2 is unsubstituted,

R^3 is selected from phosphatidic acid (PO_2-OH), derivatives of phosphatidic acid and bioisosters to phosphatic acid and derivatives thereof.

The present invention specifically provides a lipid-based drug delivery system for administration of an active drug substance selected from lysolipid derivatives, wherein the active drug substance is present in the lipid-based system in the form of a prodrug, said prodrug being a lipid derivative having (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, said prodrug furthermore being a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, whereby the active drug substance is liberated in the form of a lysolipid derivative which is not a substrate for lysophospholipase (EC 3.1.1.5).

The present invention furthermore provides a liposome drug delivery system for administration of an second drug substance, wherein the second drug substance is incorporated in the liposome system, said liposome system including lipid derivatives which has (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, where the lipid derivative furthermore is a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, so as to result in a fatty acid fragment and a lysolipid fragment, said lysolipid fragment not being a substrate for lysophospholipase (EC 3.1.1.5).

The present invention also relates to the compositions comprising the above and to the medical uses of the lipid derivatives and the drug delivery systems.

DESCRIPTION OF THE DRAWINGS

Fig. 1. Heat capacity curves obtained using differential scanning calorimetry. (a) Multilamellar (MLV) and unilamellar (LUV) liposomes made of 0.5 mM 1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL). (b) MLV and LUV liposomes made of dipalmitoylphosphatidylcholine (DPPC).

Fig. 2. Characteristic reaction time profile at 37 °C for phospholipase A_2, PLA_2, (*A. piscivorus piscivorus*) hydrolysis of unilamellar ProAEL liposomes. The PLA_2 hydrolysis reaction is monitored by intrinsic fluorescence (solid line) from the enzyme and 90° static light scattering (dashed lines) from the suspension. After adding PLA_2, at 400 sec to the equilibrated liplasome suspension a charateristic lag-time, τ, follows before a sudden increase in the catalytic activity takes place. Samples for HPLC are taken as indicated.

Fig. 3. HPLC chromatograms illustrating the effect of phospoholipase A_2 hydrolysis of liposomes composed of ProAEL. The left chromatogram shows the amount of ProAEL (100%) before phospholipase A_2 (*A. piscivorus piscivorus*) was added to the liplosome suspension. The right chromatogram shows the amount of ProAEL (37%) remaining after the lag-burst.

Fig. 4. PLA_2-controlled release of the fluorescent model drug calcein from ProAEL liposomes composed of 25 μM 1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) suspended in a 10 mM HEPES-buffer (pH = 7.5), as a function of time. 25 nM phospholipase A_2 (*A. piscivorus piscivorus*) was added at time 900 sec, the temperature was 37°C. The percentage of calcein released is determined as Release %=100 $(I_{F(t)}-I_B)/(I_T - I_B)$, where $I_{F(t)}$ is the measured fluorescence at time *t* after addition of the enzyme, I_B is the background fluorescence, and I_T is the total fluorescence measured after addition of Triton X-100 which leads to complete release of calcein by breaking up the liposomes.

Fig. 5. PLA_2-controlled release of the fluorescent model drug calcein across the target membrane, of non-hydrolysable membranes as shown in Fig. xx, as a function of time for liposomes composed of 25 μM 1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) suspended in a 10 mM HEPES-buffer (pH = 7.5). 25 nM phospholipase A_2 was added at time 0 and the temperature was 37°C. The percentage of calcein released is determined as described in Fig. 4.

Fig. 6. Hemolysis profile of normal red blood cells in the presence of liposomes composed of 1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL). The concentration that yield 50% hemolysis (H_{50}) was well above 1 mM. Hemolysis assay was performed as described by Perkins et al., 1997, *Biochimica et Biophysica Acta* **1327**, 61-

68.. Briefly, each sample was serially diluted with phosphate buffered saline (PBS), and 0.5 ml of each dilute suspension of ProAEL liposomes was mixed with 0.5 ml washed human red blood cells (RBC) [4% in PBS (v/v)]. Sample and standard were placed in a 37 incubater and agitated for 20 hours. Tubes were centrifuged at low speed (2000 g) for 10 minutes and 200 µl of the supernatant was quantitated by absorbance at 550 nm. 100 percent hemolysis was defined as the maximum amount of hemolysis obtanied from the detergent Triton X-100. The hemolysis profile in Fig. 6 shows a low hemolysis value (30 percent) for 1mM ProAEL-liposomes.

Fig. 7. Characteristic reaction time profiles at 37°C for PLA_2 (*A. piscivorus piscivorus*) hydrolysis of unilamellar LiPlaSomes incorporated with 0 and 5% DPPE-PEG_{2000} lipopolymers. The PLA_2 hydrolysis reaction is monitored by intrinsic fluorescence (solid line) from the enzyme and 90° static light scattering (dashed lines) from the suspension. After adding PLA_2 to the equilibrated liposome suspension a characteristic lag time follows before a sudden increase in the catalytic activity takes place.

Fig. 8. Describes the principle of liposomal drug targeting, release and absorption by endogenous enzymes.

Fig.9 (a). Schematic illustration of a liposomal drug-targeting principle involving accumulation of liposomal drug carriers in porous diseased tissue and subsequent release of drug and transport across the target membrane via endogeneous PLA_2 activity. (b) Schematic illustration of a molecular-based biophysical model system where the phospholipids of the carrier liposome, via the PLA_2-catalysed hydrolysis, act as prodestabilisers at the site of the carrier and as proenhancers at the site of the target. The possibility of extending the principle to include a lipid-based prodrug is also included.

Fig. 10 (a) PLA_2-controlled release of the fluorescent model drug calcein across the target membrane as a function of time for different compositions of the carrier liposome. The temperature is 37°C. In comparison with bare DPPC carriers, the rate of release of the model drug is dramatically enhanced for the polymer-coated carriers, DPPC + 2.5mol% DPPE-PEG_{2000}. A further augmentation of the rate of release is obtained if the carrier also contains a short-chain phospholipid, DCPC, which acts as a local activator for the enzyme. The percentage of calcein released is determined as:

Release $=100(I_{F(t)}-I_B)/(I_T-I_B)$, where $I_{F(t)}$ is the measured fluorescence at time *t* after addition of the enzyme, I_B is the background fluorescence, and I_T is the total fluorescence measured after addition of Triton X-100 which leads to complet release of calcein by breaking up the target liposomes. (b) PLA_2-controlled release of the fluorescent model drug calcein across the target membrane as a function of time for different temperatures. As the temperature is raised, the rate of release is enhanced due to increased activity of the enzyme induced by structural changes in the lipid bilayer substrate of the carrier liposome. In the present assay a maximum release of about 70% is achieved in all cases. The insert shows the time of 50% calcein release, $t_{50\%}$, as a function of temperature. The concentration of the target and carrier liposomes are 25 μM, and PLA_2 is added in a 25 nM concentration in a HEPES buffer with pH=7.5.

Fig. 11. Total release after 20 min of the fluorescent model drug calcein across the target membrane as a function of adding increasing amounts of lyso-palmitoyl phospholipid and palmitic acid, separately, and in a 1:1 mixture. The concentration of the target membranes is 25 μM in a HEPES buffer with pH=7.5 at a temperature of 39°C.

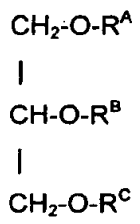
DETAILED DESCRIPTION OF THE INVENTION

One important feature of the present invention is the realisation that certain lipid derivatives will be cleaved by PLA_2 in a well-defined manner in mammalian tissue. It has been found that PLA_2 is capable of cleaving monoether/monoester lipid derivatives so as to produce monoether lysolipid derivatives which as such or in combination with other active compounds will have a therapeutic effect.

Lipid derivatives as pharmaceutically active ingredients

Thus, the present invention generally relies on lipid derivatives having (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, said lipid derivative furthermore being a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, whereby a lysolipid derivative which is not a substrate for lysophospholipase (EC 3.1.1.5) is liberated.

Although the terms "lipid" and "lysolipid" (in the context of phospholipids) will be well-known terms for the person skilled in the art, it should be emphasised that, within the present description and claims, the term "lipid" is intended to mean triesters of glycerol of the following formula:



wherein R^A and R^B are fatty acid moieties (C_{9-30}-alkyl/alkylene/alkyldiene/alkyltriene/-alkyltetraene-C(=O)-) and R^C is a phosphatidic acid (PO_2-OH) or a derivative of phosphatidic acid. Thus, the groups R^A and R^B are linked to the glycerol backbone via ester bonds.

The term "lysolipid" is intended to mean a lipid where the R^B fatty acid group is hydrolytically cleaved off, i.e. a glycerol derivative of the formula above where R^B is hydrogen, but where the other substituents are substantially unaffected. Conversion of a lipid to a lysolipid can take place under the action of an enzyme, specifically under the action of PLA_2.

The terms "lipid derivative" and "lysolipid derivative" are intended to cover possible derivatives of the above possible compounds within the groups "lipid" and "lysolipid", respectively. Examples of biologically active lipid derivatives and lysolipid derivatives are given in Houlihan, et al., Med. Res. Rev., 15, 3, 157-223. Thus, as will be evident, the extension "derivative" should be understood in the broadest sense.

Within the present application, lipid derivatives and lysolipids should however fulfil certain functional criteria (see above) and/or structural requirements. It is particularly relevant to note that the suitable lipid derivatives are those which have (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety. It will be evident that the two aliphatic groups will correspond to the two fatty acid moieties in a lipid and that the hydrophilic moiety will correspond to the phosphate part of a (phospho)lipid or a bioisoster thereof.

Thus, as the general idea behind the present invention is to exploit the increased level of PLA_2 activity in localised areas of the body of a mammal, in particular diseased tissue, the lipid derivatives which can be utilised within the present invention should be substrates for endogenous PLA_2, i.e. the lipid derivatives should be able to undergo hydrolytic, enzymatic cleavage of the aliphatic group corresponding to the fatty acid in the 2-position in a lipid. PLA_2 is known to belong to the enzyme class (EC) 3.1.1.4, thus, when reference is made to PLA_2, it should be understood that all enzymes of this class, e.g. lipases, could induce hydrolytic cleavage of the aliphatic group corresponding to the fatty acid in the 2-position in a lipid.

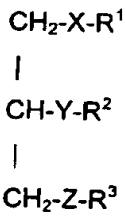
In view of the requirement to hydrolysability by PLA_2, it is clear that the aliphatic group is preferably linked via an ester or an amide functionality which can be cleaved by PLA_2, preferably so that the group which is cleaved of is a carboxylic acid.

Furthermore, it is an important feature of the present invention that the other aliphatic group (the group corresponding to the fatty acid in the 1-position in a lipid) of the lipid derivative, i.e. the lysolipid derivative after cleavage by PLA_2, is substantially unaffected by the action of PLA_2. By "substantially unaffected" is meant that the integrity of the other aliphatic group is preserved and that less than 1 mol%, preferably less than 0.1 mol%, of other aliphatic groups are cleaved under the action of PLA_2.

Also, the lysolipid derivative resulting from the hydrolytic cleavage of the aliphatic group should not in itself be a substrate for lysophospholipase. Lysophospholipase is known to belong to the enzyme class (EC) 3.1.1.5, thus, when reference is made to lysophospholipase, it should be understood that all enzymes of this class that catalysis the reaction lyso(phospho)lipid + water giving phosphoglycerol + fatty acid.

Suitable examples of such lysolipid derivatives are those which will not undergo hydrolytical cleavage under the action of lysosphospholipases, thus the lysolipid derivatives are in particular <u>not</u> lysolipids and lysolipid derivatives which have an ester linkage in the 1-position of the lysolipid or the position of a lysolipid derivative which corresponding to the 1-position of a lyoslipid and do not attack the ether or alkyl link in the 1-position.

One aspect of the invention r lates to the pharmaceutically interesting lipid derivatives which fulfil the requirement set above. One preferred class of lipid derivatives can be repr sented by the following formula:



wherein

X and Z independently are selected from O, CH_2, NH, NMe, S, S(O), and $S(O)_2$, preferably from O, NH, NMe and CH_2, in particular O;

Y is -OC(O)-, Y then being connected to R^2 via either the oxygen or carbonyl carbon atom, preferably via the carbonyl carbon atom, or Y is -NHC(O)-, Y then being connected to R^2 via either the nitrogen or carbonyl carbon atom, preferably via the carbonyl carbon atom;

R^1 is an aliphatic group of the formula Y^1Y^2;

R^2 is an aliphatic group having a length of at least 7, preferably at least 9, carbon atoms, preferably a group of the formula Y^1Y^2;

where Y^1 is $-(CH_2)_{n1}-(CH=CH)_{n2}-(CH_2)_{n3}-(CH=CH)_{n4}-(CH_2)_{n5}-(CH=CH)_{n6}-(CH_2)_{n7}-(CH=CH)_{n8r}-(CH_2)_{n9}$, and the sum of n1+2n2+n3+2n4+n5+2n6+n7+2n8+n9 is an integer of from 9 to 29; n1 is zero or an integer of from 1 to 29, n3 is zero or an integer of from 1 to 20, n5 is zero or an integer of from 1 to 17, n7 is zero or an integer of from 1 to 14, and n9 is zero or an integer of from 1 to 11; and each of n2, n4, n6 and n8 is independently zero or 1; and Y^2 is CH_3 or CO_2H; where each Y^1-Y^2 independently may be substituted with halogen or C_{1-4}-alkyl, but preferably Y^1-Y^2 is unsubstituted,

R^3 is selected from phosphatidic acid (PO_2-OH), derivatives of phosphatidic acid and bioisosters to phosphatic acid and derivatives thereof.

As mentioned above, preferr d embodiments imply that Y is -OC(O)- or -NH-C(O)- where Y is connected to R^2 via the carboxyl atom. The most preferred embodiments imply that X and Z are O and that Y is -OC(O)- where Y is connected to R^2 via the carboxyl atom. This means that the lipid derivative is a 1-monoether-2-monoester-phospholipid.

Preferably, R^1 and R^2 are aliphatic groups of the formula Y^1 Y^2 where Y^2 is CH_3 or CO_2H, but preferably CH_3, and where Y^1 is $-(CH_2)_{n1}(CH=CH)_{n2}(CH_2)_{n3}(CH=CH)_{n4}(CH_2)_{n5}-(CH=CH)_{n6}(CH_2)_{n7}(CH=CH)_{n8}(CH_2)_{n9}$; the sum of n1+2n2+n3+2n4+n5+2n6+n7+ 2n8+n9 is an integer of from 9 to 23; that is, the aliphatic group, Y^1Y^2, is from 10-24 carbon atoms in length. n1 is equal to zero or is an integer of from 1 to 23; n3 is equal to zero or is an integer of from 1 to 20; n5 is equal to zero or is an integer of from 1 to 17; n7 is equal to zero or is an integer of from 1 to 14; n9 is equal to zero or is an integer of from 1 to 11; and each of n2, n4, n6 and 8 is independently equal to zero or 1.

Although the aliphatic groups may be unsaturated and even substituted with halogens (flouro, chloro, bromo, iodo) and C_{1-4}-groups (i.e. yielding branched aliphatic groups), the aliphatic groups as R^1 and R^2 are preferably saturated as well as unbranched, that is, they preferably have no double bonds between adjacent carbon atoms, each of n2, n4, n6 and n8 then being equal to zero. Accordingly, Y^1 is preferably $(CH_2)_{n1}$. More preferably, R^1 and R^2 are each independently $(CH_2)_{n1}CH_3$, and most preferably, $(CH_2)_{17}CH_3$ or $(CH_2)_{15}CH_3$. Alternatively, the groups can have one or more double bonds, that is, they can be unsaturated, and one or more of n2, n4, n6 and n8 can be equal to 1. For example, when the unsaturated hydrocarbon has one double bond, n2 is equal to 1, n4, n6 and n8 are each equal to zero and Y^1 is $(CH_2)_{n1}$ $CH=CH(CH_2)_{n3}$. n1 is equal to zero or is an integer of from 1 to 21, and n3 is also zero or is an integer of from 1 to 20, at least one of n1 or n3 not being equal to zero.

Particularly interesting lipid derivatives are those which are monoether lipids where X and Z are O, R^1 and R^2 are independently selected from alkyl groups, $(CH_2)_nCH_3$, where n is 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29, preferably 14, 15 or 16, in particular 14; Y is -OC(O)-, Y then being connected to R^2 via the carbonyl carbon atom.

With respect to the hydrophilic moiety (often known as the "head group") which corresponds to R^3, it is believed that a wide variety of groups corresponding to

phosphatidic acid (PO_2-OH), derivatives of phosphatidic acid and bioisosters to phosphatic acid and derivatives thereof can be used. As will be evident, the crucial requirement to R^3 is that th groups should allow for enzymatic cleavage of the R^2 group by PLA_2. "Bioisosters to phosphatidic acid and derivatives thereof" indeed implies that such groups - as phosphatidic acid - should allow for enzymatic cleavage by PLA_2.

R^3 is typically selected from phosphatidic acid (PO_2-OH), phosphatidylcholine (PO_2-O-$CH_2CH_2N(CH_3)_3$), phosphatidylethanolamine (PO_2-O-$CH_2CH_2NH_2$), N-methyl-phosphatidylethanolamine (PO_2-O-$CH_2CH_2NCH_2$), phosphatidylethanolamines with a polymer covalently attached to the terminal nitrogen, and phosphatidylglycerol (PO_2-O-$CH_2CHOHCH_2OH$). Typical polymers to be covalently linked to the terminal nitrogen are polyethylene glycol (PEG), polyactide, polyglycolic acid, polyactide-polyglycolic acid copolymer, and polyvinyl alcohol, preferably those having a molecular weight from 100 Daltons to 10,000 Daltons. Other possible derivatives of phosphatidic acid are those where dicarboxylic acids, such as glutaric, sebacic, succinic and tartaric acids, are coupled to the terminal nitrogen of phosphatidylethanolamines. See also further below for additional examples of polymers and groups used to derivatise the lipid derivatives of the present invention.

One highly interesting aspect of the present invention is the possibility of modifying the pharmaceutical effect of the lipid derivative by modifying the group R^2. It should be understood that even though R^2 should be an aliphatic group having a certain length (7, preferably 9, carbon atoms), a high degree of variability is possible, e.g. R^2 need not necessarily to be a long chain residue.

Generally, it is believed that R^2 may either be rather inert for the environment in which it can be liberated by PLA_2 or that R^2 may play an active pharmaceutical role, typically as an auxiliary drug substance or as an efficiency modifier for the lysolipid derivative and/or any other (second) drug substances present in the environment.

Typically, the group R^2 will be a long chain residue, e.g. a fatty acid residue (the fatty acid will include a carbonyl from the group Y). This has been described in detail above. Interesting examples of auxiliary drug substances as R^2 within this subgroups are polyunsaturated acids, e.g. oleate, linoleic, linonleic, as well as derivatives of arachidonoyl (including the carbonyl from Y), as arachidonic acid derivatives are know regulators of

hormon action including the action of prostaglandins, thromboxanes, and leukotrines. Examples of efficiency modifiers as R^2 are those which enhance the permeability of the target cell membrane as well as enhances the activity of PLA_2 or the active drug substance or any second drug substances. Examples are short chain fatty acids.

However, it is also envisaged that other lipophilic groups might be useful as the substituent R^2, e.g. vitamin derivatives, steroid derivatives, etc. such as cholecalciferol and tocopherol analogues.

Even though it has not specifically been indicated in the general formula for the suitable examples of lipid derivatives to be used within the present invention, it should be understood that the glycol moiety of the lipid derivatives may be substituted, e.g. in order to modify the cleavage rate by PLA_2 or simply in order to modify the properties of the liposomes comprising the lipid derivatives.

As mentioned above, the present invention provides novel lipid derivatives. It should be understood that certain lipid derivatives which are similar to the one of the above formula might (coincidentally) have been described, however without realising the usefulness of such compounds. Such compounds are of course not the object of the present invention, however the various defined compositions and systems wherein such compounds are incorporated are indeed within the scope of the invention if such compositions and systems are not known or suggested for such compounds. This being said, the lipid derivative according to the invention is preferably not arachidonoyl-PAF_{16}.

The lipid derivative is preferably in the form of a particulate drug delivery system, in particular a liposome drug delivery system.

Furthermore, the present invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a lipid derivative as defined above. Preferably, the lipid derivative in such a composition is dispersed in the form of a liposome (see below).

Also, the present invention relates to such lipid derivatives for use as a medicament, preferably present in a pharmaceutical composition, and to the use of a lipid derivative as defined above for the preparation of a medicament for the treatment of diseases or

conditions associated with a localised increase in phospholipase A2 activity in mammalian tissue. Such diseases or conditions are typically selected from cancer, e.g. a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, inflammatory conditions and psoriasis. The present compositions and uses are especially applicable in the instances where the increase in PLA_2 activity is at least 25% compared to the normal level of activity in the tissue in question, the tissue being that of a mammal, in particular a human being.

Lipid derivatives as prodrugs

As described above, the present invention - in a main embodiment - i.a. provides a lipid-based drug delivery system for administration of an active drug substance selected from lysolipid derivatives, wherein the active drug substance is present in the lipid-based system in the form of a prodrug, said prodrug being a lipid derivative having (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, said prodrug furthermore being a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, whereby the active drug substance is liberated in the form of a lysolipid derivative which is not a substrate for lysophospholipase (EC 3.1.1.5). The lipid derivative is as defined above.

By the term "active drug substance" is meant any chemical entity which will provide a prophylactic or therapeutic effect in or on the body of a mammal, in particular a human being. Thus, the present invention mainly relates to the therapeutic field.

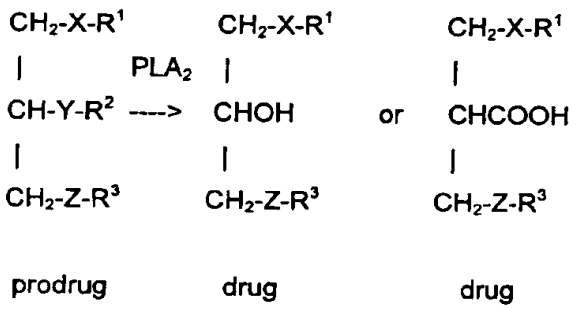
The term "prodrug" should be understood in the normal sense, namely as a drug which is masked or protected with the purpose of being converted (typically by cleavage, but also by in vivo chemical conversion) to the intended drug substance. The person skilled in the art will recognise the scope of the term "prodrug".

The active drug substance is selected from lysolipid derivatives, and as it will be understood from the present description with claims, the lysolipid derivatives relevant within the present invention will have a therapeutic effect - at least - in connection with

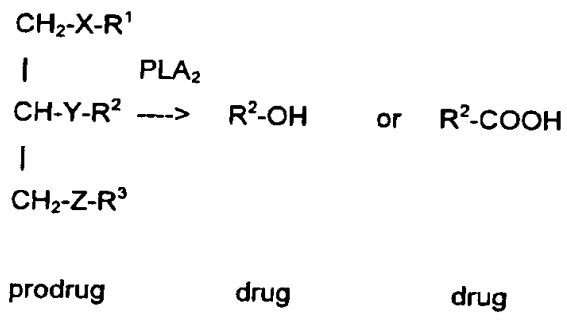
diseases and conditions where a local area of the body of the mammal has an increased level of PLA_2 activity.

As will be understood from the present description with claims, the lipid derivative will often constitute the prodrug referred to above and the lysolipid derivative will thereby constitute the active drug substance often a monoether lysolipid derivative. It should however be understood that this does not exclude the possibility of including other drug substances, referred to as second drug substances, in the drug delivery systems of the invention, neither does it exclude that the aliphatic group which can be hydrolytically cleaved by the action of PLA_2 can have a certain pharmaceutical effect (e.g. as an auxiliary drug substance or an efficiency modifier). Furthermore, the pharmaceutical effect of the "active drug substance", i.e. the lysolipid derivative, need not the be the most predominant when a second drug substance is included, actually the effect of the second drug substance might very well be the most predominant as will become apparent in the other main embodiment (see "Lipid derivative liposomes as drug delivery systems", below).

The active drug substance (lipolipid derivative) release from the prodrug (lipid derivative) is believed to take place as illustrated in the following:



Furthermore, the substituent R^2 may constitute an auxiliary drug substance or an efficiency modifier for the active drug substance and will simultaneously be released under the action of PLA_2:



It has been described above under the definition of R^2 how the group R^2 can have various independent or synergistic effects in association with the active drug substance, e.g. as an auxiliary drug substance or an efficiency modifier, e.g. permeabilit or cell lysis modifier. It should be borne in mind that the groups corresponding to R^2 (e.g. R^2-OH, R^2-COOH, or R^2-NH_2 (not shown above)) might have a pharmaceutical effect which is predominant in relation the possible effect of the lysolipid derivative (active drug substance).

Lipid derivatives formulated as liposomes

In one important variant which advantageously can be combined with the embodiments described herein, the lipid derivative (e.g. the prodrug) is included in liposomes either as the only constitutent or - which is rather common - in combination with other constituents (other lipids, sterols, etc.). Thus, the lipid-based systems described herein are preferably in the form of liposomes, wherein the liposomes are build up of layers comprising the lipid derivative (e.g. a prodrug).

"Liposomes" are known as self-assembling structures comprising one or more lipid bilayers, each of which surrounds an aqueous compartment and comprises two opposing monolayers of amphipathic lipid molecules. Amphipathic lipids (herein i.a. lipid derivatives) comprise a polar (hydrophilic) headgroup region (corresponding to the substituent R^3 in the lipid derivatives) covalently linked to one or two non-polar (hydrophobic) aliphatic groups (corresponding to R^1 and R^2 in the lipid derivatives). Energetically unfavourable contacts between the hydrophobic groups and the aqueous medium are generally believed to induce lipid molecules to rearrange such that the polar headgroups are oriented towards the aqueous medium while the hydrophobic groups reorient towards the interior of the bilayer. An energetically stable structure is formed in which the hydrophobic groups are effectively shielded from coming into contact with the aqueous medium.

Liposomes can have a single lipid bilayer (unilamellar liposomes, "ULVs"), or multiple lipid bilayers (multilamellar liposomes, "MLVs"), and can be made by a variety of methods (for a review, see, for example, Deamer and Uster, Liposomes, Marcel Dekker, N.Y., 1983, 27-52). These methods include Bangham's methods for making multilamellar liposomes (MLVs); Lenk's, Fountain's and Cullis' methods for making MLVs with substantially equal interlamellar solute distribution (see, e.g., US 4,522,803, US 4,588,578, US 5,030,453, US 5,169,637 and US 4,975,282); and Papahadjopoulos et al.'s reverse-phase evaporation method (US 4,235,871) for preparing oligolamellar liposomes. ULVs can be produced from MLVs by such methods as sonication (see Papahadjopoulos et al., Biochem. Biophys. Acta, 135, 624 (1968)) or extrusion (US 5,008,050 and US 5,059,421). The liposome of this invention can be produced by the methods of any of these disclosures, the contents of which are incorporated herein by reference.

Various methodologies, such as sonication, homogenisation, French Press application and milling can be used to prepare liposomes of a smaller size from larger liposomes. Extrusion (see US 5,008,050) can be used to size reduce liposomes, that is to produce liposomes having a predetermined mean size by forcing the liposomes, under pressure, through filter pores of a defined, selected size. Tangential flow filtration (see WO 89/08846), can also be used to regularise the size of liposomes, that is, to produce liposomes having a population of liposomes having less size heterogeneity, and a more homogeneous, defined size distribution. The contents of these documents are incorporated herein by reference. Liposome sizes can also be determined by a number of techniques, such as quasi-electric light scattering, and with equipment, e.g., Nicomp® particle sizers, well within the possession of ordinarily skilled artisans.

It is quite interesting to note that the lipid derivatives of the present invention can constitute the major part of a lipid-based system even if this system is a liposome system. This fact resides in the structural (but not functional) similarity between the lipid derivatives of the present invention and lipids. Thus, it is believed that the lipid derivatives for the present invention can be the sole constituent of liposomes, i.e. up to 100 mol% of liposomes can be constituted by the lipid derivatives. This is in contrast to the known mono-ether lysolipides, which can only constitute a minor part of liposomes.

Typically, as will be described in d tail below, liposomes advantageously include other constituents which may or may not have a pharmaceutical effect, but which will render the liposome structure more stabl (or alternatively more unstable) or will protect the liposomes against clearance and will thereby increase the circulation time thereby improving the overall efficiency of a pharmaceutical including the liposome.

This being said, it is believed that the lipid derivatives of the present invention will typically constitute from 50-100 mol%, preferably from 75-100 mol%, in particular 90-100 mol%, based on the total dehydrated liposome.

The liposomes can be unilamellar or multilamellar. Some preferred liposomes are unilamellar and have diameters of less than about 200 nm, more preferably, from greater than about 50 nm to less than about 200 nm.

The liposomes are typically - as know in the art - prepared by a method comprising the steps of: (a) dissolving the lipid derivative in an organic solvent; (b) removing the organic solvent from the lipid derivative solution of step (a); and (c) hydrating the product of step (b) with an aqueous solvent so as to form liposomes.

The method may further comprise a step of adding an second drug substance (see below) to the organic solvent of step (a) or the aqueous phase of step (c).

Subsequently, the method may comprise a step of extruding the liposomes produced in step (c) through a filter to produce liposomes of a certain size, e.g. 100 nm.

Lipid-based particulate systems, i.e. liposomes as well as micelles; of sizes covering a broad range may be prepared according to the above-mentioned techniques. Depending on the route of administration, suitable sizes for pharmaceutical applications will normally be in the range of 20-10000 nm, in particular in the range of 30-1000 nm. Sizes in the range of 50-200 nm are normally preferred because liposomes in this size range are generally believed to circulate longer in the vascular system of mammals than do larger liposomes which are more quickly recognised by the mammals' reticuloendothelial systems ("RES"), and hence, more quickly cleared from the circulation. Longer vascular circulation can enhance therapeutic efficacy by allowing more liposomes to reach their intended site of actions, e.g., tumours or inflammations.

It is believed that for a drug delivery system as defined in the embodiments herein, which are adapted to be administered via intraveneous and intramuscular injection, or via topical and ocular routes, the liposomes should preferably have a mean particle size of about 100 nm. Thus, the particle size should generally be in the range of 50-200 nm.

Furthermore, for a drug delivery system adapted to be administered via subcutaneous injection, the liposomes should preferably have a mean particle size from 100 to 5000 nm, and the liposomes can then be uni- or multilayered.

One of the advantages by including the lipid derivatives in liposomes is that the liposome structure, in particular when stabilised as described in the following, will have a much longer vascular circulation time that the lipid derivatives as discrete compounds. Furthermore, the lipid derivatives will become more or less inert or even "invisible" when "packed" in liposomes, in particular liposomes including lipopolymers. This means than any potential disadvantageous effect, e.g. hemolytic effect, can be suppressed.

The liposomes should preferably act as inert constituents until they react the area of interest, e.g. cancerous, infected or inflammatorily diseased areas or tissue. As will be described in the following, liposomes may include a number of other constituents. In particular, a drug delivery system according to the invention may further contain a component which controls the release of any second drug substance, PLA_2 activity controlling agents or permeability enhancer, e.g. short chain lipids and lipopolymers.

The liposomes' bi- or multilayers may also contain other constituents such as other lipids, sterolic compounds, polymer-ceramides, lipopolymers such as PEG-lipids, glycolipids as stabilisers and targetting compounds, etc.

The liposomes comprising lipid derivatives may (in principle) exclusively consist of the lipid derivatives. However, in order to modify the liposomes, "other lipids" may be included as well. Other lipids are selected for their ability to adapt compatible packing conformations with the lipid derivative components of the bilayer such that the all the lipid constituents are tightly packed, and release of the lipid derivatives from the bilayer is inhibited. Lipid-based factors contributing to compatible packing conformations are well known to ordinarily skilled artisans and include, without limitation, acyl chain length and

degree of unsaturation, as well as the headgroup size and charge. Accordingly, suitable other lipids, including various phosphatidylethanolamines ("PE's") such as egg phosphatidylethanolamin ("EPE") or dioleoyl phosphatidylethanolamine ("DOPE"), can be selected by ordinarily skilled artisans without undue experimentation. Lipids may be modified in various way, e.g. by headgroup derivatisation with dicarboxylic acids, such as glutaric, sebacic, succinic and tartaric acids, preferably the dicarboxylic acid is glutaric acid ("GA"). Accordingly, suitable headgroup-derivatised lipids include phosphatidyl-ethanolamine-dicarboxylic acids such as dipalmitoyl phosphatidylethanolamine-glutaric acid ("DPPE-GA"), palmitoyloleoyl phosphatidylethanolamine-glutaric acid ("POPE-GA") and dioleoyl phosphatidylethanolamine-glutaric acid ("DOPE-GA"). Most preferably, the derivatised lipid is DOPE-GA.

The total content of "other lipids" will typically be in the range of 0-30 mol%, in particular 1-10 mol%, based on the total dehydrated liposome.

Sterolic compound included in the liposome may generally affects the fluidity of lipid bilayers. Accordingly, sterol interactions with surrounding hydrocarbon groups generally inhibit emigration of these groups from the bilayer. An examples of a sterolic compound (sterol) to be included in the liposome is cholesterol, but a variety of other sterolic compounds are possible. It is generally believed that the content of sterolic compound, if present, will be in the range of 0-25 mol%, in particular 0-10 mol%, such as 0-5 mol%, based on the total dehydrated liposome.

Polymer-ceramides are stabilisers improving the vascular circulation time. Examples are polyethylene glycol derivatives of ceramides (PEG-ceramides), in particular those where the molecular weight of the polyethylene glycol is from 100 to 5000. It is generally believed that the content of polymer-ceramides, will be in the range of 0-30 mol%, in particular 0-10 mol%, based on the total dehydrated liposome.

One very important group of compounds to be included in liposomes as modifiers are stabilising compound from the group of lipopolymers (e.g. polyethyleneoxide-dipalmitoylphosphatidyl ethanolamine, DPPE-PEG, polyethyleneoxide-distearoylphosphatidylethanolamine, DSPE-PEG) with PEG molecular weight from 100 to 10000 Daltons. It has been shown that lipopolymers function as stabilisers for the liposome, i.e. lipopolymer increases the circulation time, and - which is highly interesting

in the present context, as activators for PLA_2. The stabilising effect will be described in the following.

Liposome outer surfaces are believed to become coated with serum proteins, such as opsonins, in mammals' circulatory systems. Without intending in any way to be limited by theory, it is believed that liposome clearance can be inhibited by modifying the outer surface of liposomes such that binding of serum proteins thereto is generally inhibited. Effective surface modification, that is, alterations to the outer surfaces of liposomes which result in inhibition of opsonisation and RES uptake, is believed to be accomplished by incorporating into liposomal bilayers lipids whose polar headgroups have been derivatised by attachment thereto of a chemical moiety which can inhibit the binding of serum proteins to liposomes such that the pharmacokinetic behavior of the liposomes in the circulatory systems of mammals is altered and the activity of PLA_2 is enhanced as described for the lipopolymers above.

Liposome preparations have been devised which avoid rapid RES uptake and which thus have an increased half-life in the bloodstream. STEALTH® liposomes (Liposome Technology Inc., Menlo Park, Calif.) include polyethyleneglycol (PEG)-grafted lipids at about 5 mol % in the lipid bilayer. The presence of polymers on the exterior liposome surface decreases the uptake of liposomes by the organs of the RES. The liposome membranes can be constructed so as to resist the disruptive effects of the surfactant contained therein. For example, a liposome membrane which contains as constituents lipids derivatised with a hydrophilic (i.e., water-soluble) polymer normally has increased stability. The polymer component of the lipid bilayer protects the liposome from uptake by the RES, and thus the circulation time of the liposomes in the bloodstream is extended.

Hydrophilic polymers suitable for use in lipopolymers are those which are readily water-soluble, can be covalently attached to a vesicle-forming lipid, and which are tolerated in vivo without toxic effects (i.e., are biocompatible). Suitable polymers include polyethylene glycol (PEG), polylactic (also termed polylactide), polyglycolic acid (also termed polyglycolide), a polylactic-polyglycolic acid copolymer, and polyvinyl alcohol. Preferred polymers are those having a molecular weight of from about 100 or 120 daltons up to about 5,000 or 10,000 daltons, and more preferably from about 300 daltons to about 5,000 daltons. In a particularly preferred embodiment, the polymer is polyethyleneglycol having a molecular weight of from about 100 to about 5,000 daltons, and more preferably

having a molecular weight of from about 300 to about 5,000 daltons. In a particularly preferred embodiment, the polymer is polyethyleneglycol of 750 daltons (PEG(750)). Polymers may also be defined by the number of monomers therein; a preferred embodiment of the present invention utilises polymers of at least about three monomers, such PEG polymers consisting of three monomers (approximately 150 daltons). Other hydrophilic polymers which may be suitable for use in the present invention include polyvinylpyrrolidone, polymethoxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide, polydimethylacrylamide, and derivatised celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

It is generally believed that the content of lipopolymer advantageously will be in the range of 0-50 mol%, in particular 0-15 mol%, based on the total dehydrated liposome.

Still other ingredients may constitute 0-2 mol%, in particular 0-1 mol%, based on the total dehydrated liposome.

According to an embodiment of the present invention, the lipid bilayer of a liposome contains lipids derivatised with polyethylene glycol (PEG), such that the PEG chains extend from the inner surface of the lipid bilayer into the interior space encapsulated by the liposome, and extend from the exterior of the lipid bilayer into the surrounding environment (see US 5,882,679).

A variety of coupling methods for preparing a vesicle-forming lipid derivatised with a biocompatible, hydrophilic polymer such as polyethylene glycol are known in the art. See, e.g., US 5,213,804; US 5,013,556).

The derivatised lipid components of liposomes according to the present invention may additionally include a labile lipid-polymer linkage, such as a peptide, ester, or disulfide linkage, which can be cleaved under selective physiological conditions, such as in the presence of peptidase or esterase enzymes or reducing agents. Use of such linkages to couple polymers to phospholipids allows the attainment of high blood levels of such liposomes for several hours after administration, followed by cleavage of the reversible linkages and removal of the polymer from the exterior liposome bilayer. The polymer-less liposomes are then subject to rapid uptake by the RES system. See, e.g., US 5,356,633).

Additionally, liposomes according to the present invention may contain non-polymer molecules bound to th xterior of the liposome, such as haptens, enzymes, antibodies or antibody fragments, cytokines and hormones (see, e.g., US 5,527,528), and other small proteins, polypeptides, single sugar polysaccharide moieties, or non-protein molecules which confer a particular enzymatic or surface recognition feature to the liposome. See published PCT application WO 94/21235. Surface molecules which preferentially target the liposome to specific organs or cell types are referred to herein as "targeting molecules" and include, for example, antibodies and sugar moieties, e.g. gangliosides or those based on mannose and galactose, which target the liposome to specific cells bearing specific antigens (receptors for sugar moieties). Techniques for coupling surface molecules to liposomes are known in the art (see, e.g., US 4,762,915).

The liposome can be dehydrated, stored and then reconstituted such that a substantial portion of its internal contents is retained. Liposomal dehydration generally requires use of a hydrophilic drying protectant such as a disaccharide sugar at both the inside and outside surfaces of the liposome bilayers (see US 4,880,635). This hydrophilic compound is generally believed to prevent the rearrangement of the lipids in the liposome, so that the size and contents are maintained during the drying procedure and through subsequent rehydration. Appropriate qualities for such drying protectants are that they are strong hydrogen bond acceptors, and possess stereochemical features that preserve the intramolecular spacing of the liposome bilayer components. Alternatively, the drying protectant can be omitted if the liposome preparation is not frozen prior to dehydration, and sufficient water remains in the preparation subsequent to dehydration.

Lipid derivative liposomes as drug carrier systems

As mentioned above, the liposomes including the lipid derivatives of the present invention may also include second drug substances. In particular, the lipid-based drug delivery system described above is preferably in the form of liposomes wherein a second drug substance is incorporated. It should be understood that second drug substances may comprise pharmaceutically active ingredients which may have an individual or synergistic pharmaceutical effect in combination with the lipid derivative and lysolipid derivatives. The term "second" does not necessarily imply that the pharmaceutical effect of the second drug substance is inferior in relation to that of, e.g., the active drug substance derived

from the prodrug, but is merely used to differentiate between the two groups of substances.

This being said, the present invention also provides a drug delivery system which is in the form of liposomes, and wherein a second drug substance is incorporated.

A possible "second drug substance" is any compound or composition of matter that can be administered to mammals, preferably humans. Such agents can have biological activity in mammals; the agents can also be used diagnostically in the mammals. Second drug substances which may be associated with liposomes include, but are not limited to: antiviral agents such as acyclovir, zidovudine and the interferons; antibacterial agents such as aminoglycosides, cephalosporins and tetracyclines; antifungal agents such as polyene antibiotics, imidazoles and triazoles; antimetabolic agents such as folic acid, and purine and pyrimidine analogs; antineoplastic agents such as the anthracycline antibiotics and plant alkaloids; sterols such as cholesterol; carbohydrates, e.g., sugars and starches; amino acids, peptides, proteins such as cell receptor proteins, immunoglobulins, enzymes, hormones, neurotransmitters and glycoproteins; dyes; radiolabels such as radioisotopes and radioisotope-labeled compounds; radiopaque compounds; fluorescent compounds; mydriatic compounds; bronchodilators; local anesthetics; and the like.

Liposomal second drug substance formulations enhance the therapeutic index of the second drug substances by reducing the toxicity of the drug. Liposomes can also reduce the rate at which a second drug substance is cleared from the vascular circulation of mammals. Accordingly, liposomal formulation of second drug substance can mean that less of the drug need be administered to achieve the desired effect.

Liposomes can be loaded with one or more second drug substances by solubilising the drug in the lipid or aqueous phase used to prepare the liposomes. Alternatively, ionisable second drug substances can be loaded into liposomes by first forming the liposomes, establishing an electrochemical potential, e.g., by way of a pH gradient, across the outermost liposomal bilayer, and then adding the ionisable second drug substance to the aqueous medium external to the liposome (see US 5,077,056 and WO 86/01102).

Methods of preparing lipophilic drug derivatives which are suitable for liposome or micelle formulation are known in the art (see e.g., US 5,534,499 describing covalent attachment

of therapeutic agents to a fatty acid chain of a phospholipid). A micellar formulation of taxol is described in Alkan-Onkyuksel et al., Pharmaceutical Research, 11:206 (1994).

Accordingly, the second drug substance may be any of a wide variety of known and possible pharmaceutically active ingredients, but is preferably a therapeutically and/or prophylactically active substance. Due to the mechanism involved in the degradation of the liposomes of the present invention, it is preferred that the second drug substance is one relating to diseases and/or conditions associated with a localised increase in PLA_2 activity.

Particularly interesting second drug substances are selected from (i) antitumour agents such as anthracyline derivatives, cisplatin, paclitaxel, 5-fluoruracil, and vincristine, (ii) antibiotics and antifungals, and (iii) antiinflammatory agents such as steroids and non-steroids. In particular the steroids can also have a stabilising effect on the liposomes.

The cytotoxic effects of a broad range of anticancer agents are likely to improve when encapsulated in the carriers of this invention. Furthermore, it is expected that the hydrolysis products, i.e. monoether lysolipids and ester-linked derivatives, act in turn as absorption enhancers for drug permeation across the target membranes when the carriers locally are broken down in the diseased tissue.

It is envisaged that the second drug substance will be distributed in the liposomes according to their hydrophilicity, i.e. hydrophilic second drug substances will tend to be present in the cavity of the liposomes and hydrophobic second drug substances will tend to be present in the hydrophobic bilayer. Method for incorporation of second drug substances are know in the art as has been made clear above.

An intriguing possibility within the scope of the present invention is to utilise the drug delivery system for localised deliverance of diagnostic agents, e.g. for MR or PET scanning.

It should be understood from the above, that the lipid derivatives may - as prodrugs or discrete constituents - posses a pharmaceutical activity. Disregarding this effect for a moment, the present invention furthermore relates to a general liposome drug delivery system for administration of an second drug substance, wherein the second drug

substance is incorporated in the liposome system, said liposome system including lipid derivatives which has (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, where the lipid derivative furthermore is a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, so as to result in a fatty acid fragment and a lysolipid fragment, said lysolipid fragment not being a substrate for lysophospholipase (EC 3.1.1.5).

As above for the system according to the other embodiment, the aliphatic group which can be hydrolytically cleaved off, may be an auxiliary drug substance or an efficiency modifier for the second drug substance. It should be understood that the lipid derivative is a lipid derivative as defined further above. Typically, the lipid derivative constitutes a major part of the liposome system.

As should be understood from the above, the present invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and any of the lipid-based drug delivery systems described above. The composition will be described in detail below.

The present invention also relates to the use of any of the lipid-based drug delivery systems described herein as a medicament, and to the use of any of the lipid-based drug delivery systems described herein for the preparation of a medicament for the treatment of diseases or conditions associated with a localised increase in phospholipase A2 activity in mammalian tissue. Such diseases or conditions are typically selected from cancer, e.g. a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, inflammatory conditions and psoriasis. Also included is the prophylactic use, the use as antiviral agent, use against leishmania parasite and also against multiple sclerosis. The present compositions and uses are especially applicable in the instances the increase in PLA_2 activity is at least 25% compared to the normal level of activity in the tissue in question, the tissue being that of a mammal, in particular a human being.

Pharmaceutical preparations and therapeutic uses

Also provided herewith is a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the lipid derivative, e.g. as a liposome, of this invention.

"Pharmaceutically acceptable carriers" as used herein are those media generally acceptable for use in connection with the administration of lipids and liposomes, including liposomal drug formulations, to mammals, including humans. Pharmaceutically acceptable carriers are generally formulated according to a number of factors well within the purview of the ordinarily skilled artisan to determine and account for, including without limitation: the particular active drug substance and/or second drug substance used, the liposome preparation, its concentration, stability and intended bioavailability; the disease, disorder or condition being treated with the liposomal composition; the subject, its age, size and general condition; and the composition's intended route of administration, e.g., nasal, oral, ophthalmic, topical, transdermal, vaginal, subcutaneous, intramammary, intraperitoneal, intravenous, or intramuscular. Typical pharmaceutically acceptable carriers used in parenteral drug administration include, for example, D5W, an aqueous solution containing 5% weight by volume of dextrose, and physiological saline. Pharmaceutically acceptable carriers can contain additional ingredients, for example those which enhance the stability of the active ingredients included, such as preservatives and anti-oxidants.

The liposome or lipid derivative is typically formulated in a dispersion medium, e.g. a pharmaceutically acceptable aqueous medium.

An amount of the composition comprising an anticancer effective amount of the lipid derivative, typically from about 0.1 to about 1000 mg of the lipid derivative per kg of the mammal's body, is administered, preferably intravenously. For the purposes of this invention, "anticancer effective amounts" of liposomal lipid derivatives are amounts effective to inhibit, ameliorate, lessen or prevent establishment, growth, metastasis or invasion of one or more cancers in mammals to which the lipid derivatives have been administered. Anticancer effective amounts are generally chosen in accordance with a number of factors, e.g., the age, size and general condition of the subject, the cancer being treated and the intended route of administration, and determined by a variety of means, for example, dose ranging trials, well known to, and readily practiced by, ordinarily skilled artisans given the teachings of this invention. Antineoplastic effective amounts of

the liposomal drugs/prodrugs of this invention are about the same as such amounts of free, nonliposomal, drugs/prodrugs, e.g., from about 0.1 mg of the lipid derivative per kg of body weight of the mammal being treated to about 1000 mg p r kg.

Preferably, the liposome administered is a unilamellar liposome having an average diameter of from about 50 nm to about 200 nm. The anti-cancer treatment method can include administration of one or more second drug substances in addition to the liposomal drug, these additional agents preferably, but not necessarily, being included in the same liposome as the lipid derivative. The second drug substances, which can be entrapped in liposomes' internal compartments or sequestered in their lipid bilayers, are preferably, but not necessarily, anticancer agents or cellular growth promoting factors.

The pharmaceutical composition is preferably administered parenterally by injection, infusion or implantation (intravenous, intramuscular, intraarticular, subcutaneous or the like) in dosage forms, formulations or e.g. suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants.

The formulation and preparation of such compositions is well-known to those skilled in the art of pharmaceutical formulation. Specific formulations can be found in the textbook entitled "Remington's Pharmaceutical Sciences".

Thus, the pharmaceutical compositions according to the invention may comprise the active drug substances in the form of a sterile injection. To prepare such a composition, the suitable active drug substances are dispersed in a parenterally acceptable liquid vehicle which conveniently may comprise suspending, solubilising, stabilising, pH-adjusting agents and/or dispersing agents. Among acceptable vehicles that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution and isotonic sodium chloride solution. The aqueous formulation may also contain one or more preservatives, for example, methyl, ethyl or n-propyl p-hydroxybenzoate.

The pharmaceutical compositions may also be administered topically on the skin for percutaneous absorption in dosage forms or formulations containing conventionally non-toxic pharmaceutical acceptable carriers and excipients. The formulations include creams, ointments, lotions, liniments, gels, hydrogels, solutions, suspensions, sticks, sprays,

pastes, plasters and other kinds of transdermal drug delivery systems. The pharmaceutically acceptable carriers or excipients may include emulsifying agents, antioxidants, buffering agents, preservatives, humectants, penetration enhancers, chelating agents, gelforming agents, ointment bases, perfumes and skin protective agents. Examples of emulsifying agents are naturally occurring gums, e.g. gum acacia or gum tragacanth, naturally occurring phosphatides, e.g. soybean lecithin and sorbitan monooleate derivatives. Examples of antioxidants are butylated hydroxy anisole (BHA), ascorbic acid and derivatives thereof, tocopherol and derivatives thereof, butylated hydroxy anisole and cysteine. Examples of preservatives are parabens, such as methyl or propyl p-hydroxybenzoate and benzalkonium chloride. Examples of humectants are glycerin, propylene glycol, sorbitol and urea. Examples of penetration enhancers are propylene glycol, DMSO, triethanolamine, N,N-dimethylacetamide, N,N-dimethylformamide, 2-pyrrolidone and derivatives thereof, tetrahydrofurfuryl alcohol and Azone®. Examples of chelating agents are sodium EDTA, citric acid and phosphoric acid. Examples of gel forming agents are Carbopol, cellulose derivatives, bentonite, alginates, gelatin and polyvinylpyrrolidone. Examples of ointment bases are beeswax, paraffin, cetyl palmitate, vegetable oils, sorbitan esters of fatty acids (Span), polyethylene glycols, and condensation products between sorbitan esters of fatty acids and ethylene oxide, e.g. polyoxyethylene sorbitan monooleate (Tween).

The pharmaceutical compositions mentioned above for topical administration on the skin may in particular be used in connection with topical administration onto or close to the diseased parts of the body which is to be treated. The compositions may be any suitable medicated mass adapted for direct application or for introduction into relevant orifice(s) of the body, e.g. the rectal, urethral, vaginal or oral orifices. The compositions may simply be applied directly onto the diseased part. In certain cases it might be applied by means of special drug delivery devices such as dressings or alternatively plasters, pads, sponges, strips or other forms of suitable flexible material.

The topical administration route is particularly interesting, in particular in connection with skin cancer, inflammations and psoriasis. Due to the fact that the liposome will have to cross the skin through small pores in statum corneum driven by a high water content in the deeper skin layers, it is relevant to consider to add membrane softening additives in a concentration needed to increase the bending rigidity of the prodrug-lipid membrane and make the liposomes sufficiently deformable to penetrate into and cross the skin, thus, the

technology of transfersomes (Cevc et al. (1995), J. Control. Release 36, 3-16) may advantageously be utilised.

Where treatment of a tumour or neoplasm is desired, effective delivery of a liposome-encapsulated drug via the bloodstream requires that the liposome be able to penetrate the continuous (but "leaky") endothelial layer and underlying basement membrane surrounding the vessels supplying blood to a tumour. Liposomes of smaller sizes have been found to be more effective at extravasation into tumours through the endothelial cell barrier and underlying basement membrane which separates a capillary from tumour cells.

As used herein, "solid tumours" are those growing in an anatomical site other than the bloodstream (in contrast to blood-borne tumours such as leukemias). Solid tumours require the formation of small blood vessels and capillaries to nourish the growing tumour tissue.

In accordance with the present invention, the anti-tumour or anti-neoplastic agent of choice is entrapped within a liposome according to the present invention; the liposomes are formulated to be of a size known to penetrate the endothelial and basement membrane barriers. The resulting liposomal formulation can be administered parenterally to a subject in need of such treatment, preferably by intravenous administration. Tumours characterised by an acute increase in permeability of the vasculature in the region of tumour growth are particularly suited for treatment by the present methods. The liposomes will eventually degrade due to lipase action at the tumour site, or can be made permeable by, for example, thermal or ultrasonic radiation. The drug is then released in a bioavailable, transportable solubilised form. Furthermore, a small elevation in temperature as often seen in diseased tissue may further increase the stimulation of PLA_2.

Where site-specific treatment of inflammation is desired, effective liposome delivery of an drug requires that the liposome have a long blood half-life, and be capable of penetrating the continuous endothelial cell layer and underlying basement membrane surrounding blood vessels adjacent to the site of inflammation. Liposomes of smaller sizes have been found to be more effective at extravasation through the endothelial cell barrier and into associated inflamed regions. However, the limited drug-carrying capacity of conventional small liposome preparations has limited their effectiveness for such purposes.

In accordance with the present invention, the anti-inflammatory agent of choice is entrapped within a liposome according to the present invention; the liposomes are formulated to be of a size known to penetrate the endothelial and basement membrane barriers. The resulting liposomal formulation can be administered parenterally to a subject in need of such treatment, preferably by intravenous administration. Inflamed regions characterised by an acute increase in permeability of the vasculature in the region of inflammation are particularly suited for treatment by the present methods.

It is known that the activity of PLA_2 is abnormally high in areas of the mammalian body diseased by cancer, inflammation, psoriasis, etc. The present invention have provided a way of exploiting this fact, and it is believed that the PLA_2 activity should be at least 25% higher in the diseases area of the body (determined in the extracellular environment) compared with a comparative normal area. It is however envisaged that the level of PLA_2 activity often is much higher, e.g. at least 100%, e.g. at least 200% such as at least 400%. This means that treatment of a mammal in need of a treatment with the purpose of cure or relief, can be conducted with only minimal influence on tissue having a "normal" level of PLA_2 activity. This is extremely relevant in particular with the treatment of cancer where rather harsh drug (second drug substances) are often needed.

Residing in the realisations behind the present invention, the invention thus provides to a method for selectively drug targeting to diseased areas, such as areas comprising neoplastic cells, e.g., areas within the mammalian body, preferably a human, having a phospholipase A2 (PLA_2) activity which is at least 25% higher compared to the normal activity in said areas, by administering to the mammal in need thereof an efficient amount of a drug delivery system defined herein.

Provided is also a method of treating of a mammal afflicted with a cancer, e.g., a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, which comprises administering a pharmaceutical composition of this invention to the mammal. It is believed that the lipid derivatives and/or second drug substance in liposome form is selectively cytotoxic to tumour cells.

Toxicity

Toxicity of the liposomes comprising the lipid derivatives can be assessed by determining the therapeutic window "TW," which is a numerical value derived from the relationship between the compound's induction of hemolysis and its ability to inhibit the growth of tumour cells. TW values are determined in accordance with the formula HI_5 /GI_{50} (wherein "HI_5" equals the concentration of compound inducing the hemolysis of 5% of the red blood cells in a culture, and wherein "GI_{50}" equals the dose of compound inducing fifty percent growth inhibition in a population of cells exposed to the agent). The higher an agent's HI_5 value, the less hemolytic is the agent - higher HI_5 's mean that greater concentrations of compound are required to be present in order for the compound to induce 5% hemolysis. Hence, the higher its HI_5, the more therapeutically beneficial is a compound, because more of it can be given before inducing the same amount of hemolysis as an agent with a lower HI_5. By contrast, lower GI_{50} 's indicate better therapeutic agents - a lower GI_{50} value indicates that a lesser concentration of an agent is required for 50% growth inhibition. Accordingly, the higher is its HI_5 value and the lower is its GI_{50} value, the better are a compound's agent's therapeutic properties.

Generally, when a drug's TW is less than 1, it cannot be used effectively as a therapeutic agent. That is, the agent's HI_5 value is sufficiently low, and its GI_{50} value sufficiently high, that it is generally not possible to administer enough of the agent to achieve a sufficient level of tumour growth inhibition without also attaining an unacceptable level of hemolysis. As the lipid derivative liposomes take advantage of the lower PLA_2 activity in the bloodstream compared to the activity in the diseased tissue, it is believed that the TW will be much higher that for normal monoether lysolipids. As the variance in activity is in orders of magnitude and as the liposomes will be "trapped" in tissue with a high PLA_2 activity, it is generally believed the TW of the liposomes of the invention will be greater than about 3, more preferably greater than about 5, and still more preferably greater than about 8.

The invention will be illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Liposome preparation

Unilamellar fully hydrated liposomes with a narrow size distribution were made from

1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) and di-hexadecanoyl-*sn*-glycero-3-phosphocholine (dipalmitoylphosphatidylcholine, DPPC). The lipids were obtained from Sigma Chemical Co. and Avanti Polar Lipids, respectively. Briefly, weighed amounts of DPPC and ProAEL were dissolved in chloroform. The solvent was removed by a gentle stream of N_2. The lipid films were then dried overnight under low pressure to remove trace amounts of solvent. Multilamellar vesicles were made by dispersing the dried lipids in a buffer solution containing: 150 mM KCL, 10 mM HEPES (pH = 7.5), 1 mM NaN_3, 30 μM $CaCl_2$ and 10 μM EDTA. The multilamellar vesicles were extruded ten times through two stacked 100 nm pore size polycarbonate filters as described by Mayer et al., *Biochim. Biophys. Acta*, **858**, 161-168.

Heat capacity curves were obtained using a N-DSC II differential scanning calorimetry (Calorimetry Sciences Corp., Provo) of the power compensating type with a cell volume of 0.34 mL Before scanning the liposome suspension was equilibrated for 50 min in the calorimeter at the starting temperature. A scan rate of +10 °C/h was used. The lipid concentration was 0.5 mM for the ProAEL liposomes and 1 mM for the DPPC-liposomes. The gel-to-fluid transition of the multilamellar liposomes (MLV) is characterised as a sharp first-order transition, as reflected by the narrow peak in the heat capacity curves shown in Figs. 1a and 1b (upper curves) for DPPC and ProAEL multilamellar liposomes. The sharp peak reflecting the transitional behaviour of multilamellar liposomes is in contrast to the broader gel-to-fluid transition observed for unilamellar liposomes (LUV) (Pedersen et al., 1996, *Biophys. J.* **71**, 554-560) as shown in Figs. 1a and 1b (lower curves) for the unilamellar extruded DPPC and ProAEL liposomes

Example 2

Phospholipase A_2 reaction profile and lag time measurements

Purified snake-venom phospholipase A_2 (PLA_2 from *Agikistrodon piscivorus piscivorus*) has been isolated according to the procedure of Maraganore et al., *J. Biol. Chem.* **259**, 13839-13843. This PLA_2 enzyme belongs to the class of low-molecular weight 14kD secretory enzymes which display structural similarity to human extracellular phospholipase A_2 indicating a common molecular mechanisms of the phospholipase catalysed hydrolysis at the lipid-membrane interface (Wery et al., *Nature* **352**, 79-82; Hønger et al. *Biochemistry* **35**, 9003-9006; Vermehren et al., *Biochimica et Biophysica Acta* **1373**, 27-36). Unilamellar fully hydrated liposomes with a narrow size distribution were prepared from

1-O-hexadecyl-2-h xadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) as described above.

Assay conditions for the PLA_2 reaction time profile shown in Fig. 2 and the lag time measurements reported in Table 1 were 0.15 mM unilamellar ProAEL-liposomes, 150 nM PLA_2, 150 mM KCL, 10 mM HEPES (pH 7.5), 1 mM NaN_3, 30 μM $CaCl_2$, and 10 μM EDTA.

Temp. (°C)	Lag-time τ (sec.)	% ProAEL hydrolyzed
39	32	61.2
37	10	75.4
31	100	53.4

Table 1. Lag-time, τ, and percent hydrolysed ProAEL at different temperatures, determined by HPLC. The concentration of 1-O-Hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) was 0.150 mM in a 10 mM HEPES-buffer (pH = 7.5). 150 nM PLA_2 (*A. piscivorus piscivorus)* was added after an equilibration time of 20 minutes.

The catalytic reaction was initiated by adding 8.9 μL of a 42 μM PLA_2 stock solution to 2.5 ml of the thermostated liposome suspension equilibrated for at least 20 min prior to addition of PLA_2. The characteristic lag-burst behaviour of PLA_2 towards liposome is determined by a sudden increase in the intrinsic fluorescence from PLA_2 at 340 nm after excitation at 285 nm followed by a concomitant decrease in the 90° light scattering from the lipid suspension (Hønger et al., *Biochemistry* **35**, 9003-9006). Samples for HPLC analysis of the amount of non-hydrolysed ProAEL remaining and consequently the amount of AEL generated were taken as marked by the vertical arrows in Fig. 2. The HPLC chromatograms in Fig. 3 show the amounts of ProAEL before (t = 300 sec) and after (t = 1300 sec) the addtion of PLA_2 at t = 400 sec to the ProAEL-liposome suspension. HPLC analysis was made using a 5 μm diol column, a mobile phase composed of chloroform/methanol/water (730:230:30, v/v) and an evaporative light scattering detector. The turnover of the PLA_2 catalysed lipid hydrolysis of ProAEL to AEL was measured by quantitative HPLC at different temperatures (see Table 1), simultaneously with the collection of intrinsic enzyme fluorescence and 90 °C light scattering. 100 μl aliquots were withdrawn from the lipid suspension and rapidly mixed with 1 ml chloroform/methanol/acetic acid (2:4:1) solution in order to quench the

enzymatic reaction. The solution was washed with 1 ml of water and 20 µl of the heavy organic phase was used for HPLC.

Example 3

Phospholipase A_2 induced release of an incapsulated water-soluble model drug

Multilamellar ProAEL-liposomes in the presence of fluorescent calcein in a self-quenching concentration of 20 mM were made by hydrating a film of ProAEL in a HEPES buffer solution at pH=7.5 for one hour at 10 °C above the phase transition temperature. Unilamellar liposomes were made by extruding the multilamellar liposomes ten times through two stacked 100 nm polycarbonate filters. The unilamellar liposomes were rapidly cooled to a temperature below the transition temperature, and the calcein-containing ProAEL liposomes were separated from free calcein using a chromatographic column packed with Sephadex G-25.

Assay conditions for the PLA_2 induced calcein release were 0.025 mM unilamellar ProAEL-liposomes, 25 nM PLA_2, 150 mM KCL, 10 mM HEPES (pH 7.5 or 8.0), 1 mM NaN_3, 30 µM $CaCl_2$, and 10 µM EDTA. PLA_2 was added at time 900 sec to 2.5 ml of the thermostated ProAEL-liposome suspension equilibrated for at least 20 min at 37 prior to addition of PLA_2. The assay of PLA_2 induced release of an incapsulated water-soluble model drug is schematically in Fig.10.b. The percentage of calcein released is determined as: % Release = 100 $(I_{F(t)}-I_B)/(I_T - I_B)$, where $I_{F(t)}$ is the measured fluorescence at time t after addition of the enzyme, I_B is the background fluorescence, and I_T is the total fluorescence measured after addition of Triton X-100 which leads to complete release of calcein by breaking up the ProAEL-liposomes. PLA_2 induced at total release of 90 percent of the entrapped calcein in the ProAEL-liposomes as shown in Fig. 4.

Example 4

Phospholipase A_2 controlled permeability increase of a target model membrane

Multilamellar model membrane target liposomes in the presence of fluorescent calcein in a self-quenching concentration of 20 mM were made by hydrating a film of 1,2-O-dioctadecyl-*sn*-glycero-3-phosphatidylcholines (D-O-SPC) in a HEPES buffer solution at pH=7.5 for one hour at 10°C above the phase transition temperature (T_m=55°C). Unilamellar liposomes were made by extruding the multilamellar liposomes ten times through two stacked 100 nm polycarbonate filters. The unilamellar liposomes were rapidly cooled to a temperature below the transition temperature, and the calcein-containing

liposomes were separated from free calcein using a chromatographic column packed with Sephadex G-25. The unilamellar carrier liposomes composed of ProAEL were prepared as described above. Calcein r lease from the target liposomes is determined by measuring the fluorescent intensity at 520 nm after excitation at 492 nm. All measurements are performed at temperatures where the lipids of both the ProAEL liposome and D-O-SPC target liposomes are in the gel state.

The concentrations of D-O-SPC and ProAEL liposomes were 25 μM. Snake venom PLA_2 (*Agkistrodon piscivorus piscivorus*) was added (25 nM) to initiate the hydrolytic reaction leading to the formation of AEL and fatty acid hydrolysis products. As calcein is released from the D-O-SPC liposomes, due to the incorporation of the non-bilayer forming AEL and fatty acid hydrolysis products into the target lipid membrane, a linear increase in the fluorescence at 520 nm after excitation at 492 nm is observed when calcein is diluted into the surrounding buffer medium as shown in Fig. 5. The percentage of calcein released is determined as described above (see Example 3).

Example 5

Hemolysis assay

Unilamellar fully hydrated liposomes with a narrow size distribution were prepared from 1-O-hexadecyl-2-hexadecanoyl-*sn*-glycero-3-phosphocholine (ProAEL) as described above.

Hemolysis assay was performed as described by Perkins et al., *Biochim. et Biophys. Acta* **1327**, 61-68. Briefly, each sample was serially diluted with phosphate buffered saline (PBS), and 0.5 ml of each dilute suspension of ProAEL liposomes was mixed with 0.5 ml washed human red blood cells (RBC) [4% in PBS (v/v)]. For controls, 0.5 ml of the red blood cell suspension was mixed with either 0.5 ml buffer solution (negative hemolysis control) or 0.5 ml water (positive hemolysis control). Sample and standard were placed in a 37 incubator and agitated for 20 hours. Tubes were centrifuged at low speed (2000 g) for 10 minutes to pellet RBCs. 200 μl of the supernatant was quantitated by absorbance at 550 nm using a Perkin-Elmer 320 Scanning Spectrophotometer. 100 percent hemolysis was defined as the maximum amount of hemolysis obtained from the detergent Triton X-100. The hemolysis profile in Fig. 6 shows a low hemolysis value (30 percent) for 1mM ProAEL-liposomes.

Example 6

Enhancement of phosph lipas A2 activity by PEG polym r graft d lipids

Unilamellar fully hydrated liposomes with a narrow size distribution were made from various concentrations of stearoyloleoylphosphatidylcholine (SOPC) and polyethyleneoxide-distearoylphosphatidylethanolamine (DSPE-PEG_{750}, MW(PEG) = 750) from Avanti Polar Lipids as described above.

Assay conditions for the PLA_2 lag-time measurements were 0.15 mM unilamellar liposomes, 150 nM PLA_2, 150 mM KCL, 10 mM HEPES (pH 7.5), 1 mM NaN_3, 30 μM $CaCl_2$, and 10 μM EDTA. The catalytic reaction was initiated by adding 8.9 μL of a 42 μM PLA_2 stock solution to 2.5 ml of the thermostated liposome suspension equilibrated for at least 20 min at 31 °C prior to addition of PLA_2. The time elapsed before the onset of rapid enzymatic activity is determined by a sudden increase in the intrinsic fluorescence from PLA_2 at 340 nm after excitation at 285 nm. The results shown in Fig. 7 show a significant decrease in the lag time when 5 mol% of DSPE-PEG_{750} (Jørgensen et al., *Pharm. Res.* **16**, 1491-1493) is incorporated into the SOPC liposomes.

Example 7

Model examples

Polymer-coated ("Stealth") liposomes can act as versatile drug-delivery systems due to long vascular circulation time and passive targeting by leaky blood vessels in diseased tissue. In the examples herein are described an experimental model system illustrating a new principle for improved and programmable drug-delivery which takes advantage of an elevated activity of endogeneous phospholipase A_2 at the diseased target tissue. The phospholipase A_2 hydrolyses a lipid-based proenhancer in the carrier liposome, producing lyso-phospholipid and free fatty acid, which are shown in a synergistic way to lead to enhanced liposome destabilisation and drug release at the same time as the permeability of the target membrane is enhanced. The proposed system can be made thermosensitive and offers a rational way for developing smart liposome-based drug delivery systems by incorporating into the carrier specific lipid-based proenhancers, prodestabilisers or prodrugs that automatically become activated by phospholipase A_2 only at the diseased target sites, such as inflammed or cancerous tissue.

Drug-delivery systems based on liposomal carriers in the 100 nm range are one of the modern microcarrier therapeutic systems that hold a promise for coming close to realising Paul Erlich's early vision of a "magic bullet" for treatment of diseases. Liposomes made of biocompatible, non-toxic phospholipids provide a system for efficient formulation and encapsulation of toxic drugs which effectively can evade the immune system.

The drug assumes the altered pharmacokinetics of the liposomal carrier and can in principle be targeted to the diseased tissue by using a combination of physico-chemical and pathophysiological factors at the sites of the liposome carrier and the target membrane, respectively. Liposomes incorporated with lipopolymers, such as polyethylene-glycol (PEG)-lipids, known as "Stealth" liposomes, display an improved stability in the vascular system, possibly due to steric protection caused by the polymer coating. The prolonged circulation time of these liposomes combined with increased vascular porosity of diseased tissue, have formed the basis for positive clinical results for specific systems, including anticancer drugs like doxorubicin as well as antibacterial and anti-inflammatory drugs.

Liposomes are self-assembled lipid systems and their stability is therefore to a large extent controlled by non-specific physical interactions. Insight into the molecular control of the physical properties of liposomes is therefore important for manipulating and tailoring the liposomal properties in relation to specific drug-delivery purposes. As an example, the thermally induced gel-fluid lipid phase transition has been exploited and optimised design systems for enhanced release of drugs due to hyperthermia. Recently, programmable fusogenic PEG-liposomes containing the anticancer drug mitoxantrone have been constructed using a time-delayed release of bilayer-stabilising lipids of the liposomes which are accumulated at the tumour sites by extravasation. It would be desirable if an intelligent and versatile drug-delivery system could be designed which has built in a dual virtual trigger mechanism of simultaneous (i) enhanced drug release selectively at the target tissue and (ii) enhanced transport of the drug into the diseased cells. This principle is illustrated schematically in Fig 9.a.

By the examples herein is described the development of a simple and operative experimental biophysical model system which sustains such a dual mechanism to be triggered at the pathological target sites. The model assumes elevated activity of endogeneous phospholipase A_2 at the diseased sites as is the case in inflammed and

cancerous tissue where the l vel of extracellular PLA_2 can be manifold magnified. Upon exposure to PLA_2, the phospholipids of the PEG-liposomes have been shown to suffer enhanced hydrolysis compared to conventional bare liposomes. This leads to destabilisation of the "Stealth" liposome and enhanced release of the encapsulated drug. The hydrolysis products, lyso-phospholipids and free fatty acids, act in turn as absorption enhancers for drug permeation across the target membrane. In this way the phospholipids of the carrier liposome behave as prodestabilisers at the site of the carrier and as proenhancers at the site of the target membrane. Molecular details of this principle are illustrated schematically in Fig. 9.b.

The experimental model system consists of a polymer-coated liposome carrier and a model target membrane. The carrier is a 100 nm unilamellar liposome made of dipalmitoyl phosphatidylcholine lipids (DPPC) with 2.5 mol% lipopolymer of the type dipalmitoyl phosphatidylethanolamine (DPPE)-PEG_{2000}. The target membrane is another liposome made of 1,2-O-dioctadecyl-sn-glycero-phosphatid\-ylcholine (D-O-SPC) which is a phospholipid where the acyl linkages of the stearoyl chains are ether bonds. In contrast to DPPC, D-O-SPC is inert towards PLA_2-catalysed hydrolysis thereby mimicking the stability of an intact target cell membrane toward degradation by its own enzymes. This experimental assay, which permits simultaneous as well as separate investigation of the effect of destabilisers at the carrier liposomes and the effect of enhancers at the target membrane, involves entrapment of a water-soluble fluorescent calcein model drug in a self-quenching concentration, in the interior of the non-hydrolysable target liposome, rather than in the carrier liposome. The enhanced level of PLA_2 at the target membrane can then be simulated by adding PLA_2 to initiate the hydrolytic reaction in a suspension of the carrier and target liposomes. The permeation of calcein across the D-O-SPC target membrane is subsequently monitored by the increase in fluorescence. In order to investigate the effect of the presence of the PEG-lipids in the carrier liposome, a similar experiment was performed with conventional bare DPPC liposomes. Furthermore, in order to compare and discriminate the permeability enhancing effect of lyso-phospholipids from that of free fatty acids, experiments without enzymes were carried out where lyso-phospholipids and free fatty acids were added simultaneously or separately to the target liposomes.

In Fig. 10.a are shown the results for the release of calcein as a function of time after adding PLA_2 to the system. The reaction time-course of the particular PLA_2 used has a

characteristic lag-burst behaviour with a so-called lag time which conveniently can be used as a measure of the enzymatic activity. A dramatic decrease in the lag time and a concomitant nhancem nt of the rat of rel ase are observed when the carrier liposomes contain the lipopolymers, DPPE-PEG_{2000}, in accordance with previous findings of enhanced PLA_2 degradation of polymer-coated liposomes.

These results suggest that the products of the PLA_2-catalysed hydrolysis of the DPPC lipids of the carrier, lyso-phospholipid and free fatty acid, which are produced in a 1:1 mixture, are incorporated into the target membrane, leading to a large increase in membrane permeability. These products, which have very low water solubility, are known, due to their non-cylindrical molecular shapes, to induce a curvature stress field in the membrane or small-scale lateral phase separation which induce membrane defects and increased permeability. This is substantiated by the data in Fig. 11 which show that the addition of lyso-phospholipid or fatty acid separately to the present target system, in the absence of PLA_2, leads to an increased rate of calcein release across the target membrane. However, the crucial finding is that if lyso-phospholipid and free fatty acid are added simultaneously in a 1:1 mixture, a dramatic enhancement in the rate of release is observed as shown in Fig. 3. This strongly suggests that the two enhancers act in a synergistic fashion, thereby highlighting the unique possibility in exploiting PLA_2-catalysed hydrolysis for combined destabilisation of the carrier liposome and enhancement of drug transport across the target membrane. The synergistic effect is further augmented by the fact that PLA_2 is activated by its own hydrolysis products revealing the degradable phospholipids of the carrier liposome as a kind of proactivators.

It should be pointed out that the effect in the present drug-delivery model system of using lipids as proenhancers and prodestabilisers via PLA_2 activity is dynamic and refers to an intrinsic time scale. This time scale is the effective retention time of the carrier liposomes near the target membrane. The more rapidly the enzyme becomes active, the faster is the drug release and the larger the drug absorption during the time which the carrier spends near the target. Furthermore, the faster the enzyme works the more readily it becomes available for hydrolysis of other drug-carrying liposomes that approach the diseased target site. Once it has been established that PLA_2 activity can be used to control drug release, several rational ways open up for intelligent improvements of the proposed drug-delivery system via use of well-known mechanisms of altering PLA_2 activity by manipulating the physical properties of the lipid bilayer to which the enzyme is known to

be sensitive. Hence the strategy is to modify certain physical properties of the carrier liposomes without significantly changing their vascular circulation time. We shall illustrate this general principle by d monstrating the effects of both a physico-chemical factor, the lipid composition of the carrier, and an enviromental (thermodynamic) factor, the local temperature at the target site.

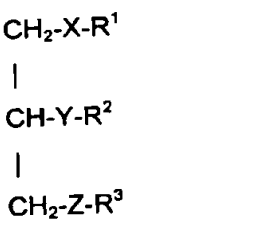
Short-chain phospholipids, such as didecanoyl phosphatidylcholine (DCPC), activate PLA_2. The effect on calcein permeation across the target membranes induced by incorporation of a small amount of DCPC into the carrier PEG-liposomes is also shown in Fig. 10.a. The release is very fast due to an almost instantaneous activation of the enzyme. We have furthermore found that PLA_2 becomes deactivated (data not shown) when a large amount of cholesterol (≈20 mol %) is incorporated into "Stealth" liposomes. In contrast we find that a small amount of cholesterol (≈3 mol%) strongly activates PLA_2. These significant findings are of particular interest since the blood circulation time of PEG-liposomes has been reported to be almost the same without cholesterol as with large amounts of cholesterol.

Temperature is known to have a dramatic and highly non-linear effect on PLA_2 activation in the region of the gel-fluid phase transition of saturated phospholipid bilayers. This effect is not caused by changes in the enzyme but by dramatic lateral structural changes in the lipid bilayer. It is possible to take advantage of this effect in the present drug-delivery system as suggested by the data in Fig. 10.b. As the temperature approaches the transition temperature at 41°C, the rate of calcein release is progressively enhanced as quantified by the time of 50% calcein release, $t_{50\%}$, shown in the insert to Fig. 10.b. It has previously been suggested that hypertermia could be exploited to enhance drug release, and that local heating at predefined tumour areas could be used to locally destabilise drug-carrying liposomes, by exploiting the enhanced leakiness of liposomes at their phase transition. In the new model drug-delivery system proposed here, these thermosensitive possibilities are integrated and fully exploited via the thermal sensitivity of PLA_2 to the physical properties of the carrier liposome. In contrast to the case where the thermic effect can only be achieved by a local temperature increase using external heating sources at a predetermined tumour site of some minimal size, the PLA_2-controlled release will be enhanced everywhere where temperature and PLA_2 concentration are elevated, e.g. in inflammed tissue, independent of the size of the diseased region and without requiring a preceding localisation of the diseased tissue.

DPPC, DCPC, D-O-SPC, and DPPE-PEG_{2000} were obtained from Avanti Polar Lipids. The DPPE-PEG_{2000} lipopolym r contains 45 monomers in the PEG polymer chain. Purified snake venom PLA_2 (*Agkistrodon piscivorus piscivorus*) was a generous gift from dr. R. L. Biltonen. This PLA_2 enzyme belongs to the class of low-molecular weight, 14kD secretory enzymes which display structural similarity to human extracellular phospholipase A_2. Multilamellar target liposomes in the presence of fluorescent calcein in a self-quenching concentration of 20mM were made by hydrating a film of D-O-SPC in a HEPES buffer solution at pH=7.5 for one hour at 10°C above the phase transition temperature T_m=55°C. Unilamellar liposomes were made by extruding the multilamellar liposomes ten times through two stacked 100nm polycarbonate filters. The unilamellar liposomes were rapidly cooled to a temperature below the transition temperature, and the calcein-containing liposomes were separated from free calcein using a chromatographic column packed with Sephadex G-25. The unilamellar carrier liposomes of DPPC, DCPC and DPPE-PEG_{2000} were prepared in a similar fashion T_m=41°C). Calcein release from the target liposomes is determined by measuring the fluorescent intensity at 520nm after excitation at 492nm. All measurements are performed at temperatures where the lipids of both the carrier and target liposomes are in the gel state.

CLAIMS

1. A lipid derivative of the following formula:



wherein

X and Z independently are selected from O, CH_2, NH, NMe, S, S(O), and $S(O)_2$, preferably from O, NH, NMe and CH_2, in particular O;

Y is -OC(O)-, Y then being connected to R^2 via either the oxygen or carbonyl carbon atom, preferably via the carbonyl carbon atom, or Y is -NHC(O)-, Y then being connected to R^2 via either the nitrogen or carbonyl carbon atom, preferably via the carbonyl carbon atom;

R^1 is an aliphatic group of the formula Y^1Y^2;

R^2 is an aliphatic group having a length of at least 7, preferably at least 9, carbon atoms, preferably a group of the formula Y^1Y^2;

where Y^1 is $-(CH_2)_{n1}-(CH=CH)_{n2}-(CH_2)_{n3}-(CH=CH)_{n4}-(CH_2)_{n5}-(CH=CH)_{n6}-(CH_2)_{n7}-(CH=CH)_{n8}-(CH_2)_{n9}$, and the sum of n1+2n2+n3+2n4+n5+2n6+n7+2n8+n9 is an integer of from 9 to 29; n1 is zero or an integer of from 1 to 29, n3 is zero or an integer of from 1 to 20, n5 is zero or an integer of from 1 to 17, n7 is zero or an integer of from 1 to 14, and n9 is zero or an integer of from 1 to 11; and each of n2, n4, n6 and n8 is independently zero or 1; and Y^2 is CH_3 or CO_2H; where each Y^1-Y^2 independently may be substituted with halogen or C_{1-4}-alkyl, but preferably Y^1-Y^2 is unsubstituted,

R^3 is selected from phosphatidic acid (PO_2-OH), derivatives of phosphatidic acid and bioisosters to phosphatic acid and derivatives thereof.

2. A lipid derivative according to claim 1, which is in the form of a liposome.

3. A lipid derivative according to any of the preceding claims, wherein X and Z are O and Y is -OC(O)-, where Y is connected to R^2 via the carboxyl atom.

4. A lipid derivative according to any of the preceding claims, wherein X and Z are O, R^1 and R^2 are independently selected from alkyl groups, $(CH_2)_nCH_3$, where n is 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29, preferably 14 or 16; Y is -OC(O)-, Y then being connected to R^2 via the carbonyl carbon atom.

5. A lipid derivative according to any of the preceding claims, wherein R^3 is selected from phosphatidic acid (PO_2-OH), phosphatidylcholine (PO_2-O-$CH_2CH_2N(CH_3)_3$), phosphatidylethanolamine (PO_2-O-$CH_2CH_2NH_2$), N-methyl-phosphatidylethanolamine (PO_2-O-$CH_2CH_2NCH_2$), phosphatidylethanolamines with a polymer covalently attached to the terminal nitrogen, and phosphatidylglycerol (PO_2-O-$CH_2CHOHCH_2OH$).

6. A lipid derivative according to any of the preceding claims, which is not arachidonoyl-PAF_{16}.

7. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the lipid derivative according to claim 1.

8. A pharmaceutical composition according to claim 7, wherein the lipid derivative is dispersed in the form of a liposome.

9. A pharmaceutical composition according to claim 8, wherein the lipid derivative constitutes 50-100 mol% of the dehydrated liposome.

10. A lipid derivative as defined in any of the claims 1-6 for use a medicament.

11. A lipid derivative according to claim 10, said lipid derivative being present in a pharmaceutical composition according to any of the claims 7-9.

12. The use of a lipid derivative as defined in any of the claims 1-6 for the preparation of a medicament for the treatment of diseases or conditions associated with a localised increase in phospholipase A2 activity in mammalian tissue.

13. The use according to claim 12, wherein the diseases or conditions are selected from cancer, e.g. a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, inflammatory conditions and psoriasis.

14. The use according to claim 12 or 13, wherein the increase in phospholipase A2 activity is a least 25% compared to the normal level of activity in the tissue in question.

15. The use according to any of the claims 12-14, wherein the lipid derivative is present in a pharmaceutical composition according to any of the claims 7-9.

16. A lipid-based drug delivery system for administration of an active drug substance selected from lysolipid derivatives, wherein the active drug substance is present in the lipid-based system in the form of a prodrug, said prodrug being a lipid derivative having (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, said prodrug furthermore being a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, whereby the active drug substance is liberated in the form of a lysolipid derivative which is not a substrate for lysophospholipase (EC 3.1.1.5).

17. A drug delivery system according to claim 16, wherein the aliphatic group which can be hydrolytically cleaved off, is an auxiliary drug substance or an efficiency modifier for the active drug substance.

18. A drug delivery system according to claim 16 or 17, wherein the prodrug is a lipid derivative as defined in any of the claims 1-6.

19. A drug delivery system according to any of the claims 16-18, wherein the prodrug constitutes the major part of the lipid-based system.

20. A drug delivery system according to any of the claims 16-19, wherein the lipid-based system is in the form of liposomes and wherein the liposomes are build up of layers comprising the lipid derivative (prodrug).

21. A drug delivery system according to any of the claims 16-20, which is in the form of liposomes, and wherein a second drug substance is incorporated.

22. A drug delivery system according to claim 21, wherein the second drug substance is a therapeutically and/or prophylactically active substance, in particular selected from (i) antitumor agents such as anthracyline derivatives, cisplatin, paclitaxel, 5-fluoruracil, and vincristine, (ii) antibiotics and antifungals, and (iii) antiinflammatory agents such as steroids and non-steroids.

23. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the lipid-based drug delivery system according to any of the claims 16-22.

24. The use of a lipid-based drug delivery system according to any of the claims 16-22 as a medicament.

25. The use of a lipid-based drug delivery system according to any of the claims 16-22 for the preparation of a medicament for the treatment of diseases or conditions associated with a localised increase in phospholipase A2 activity in mammalian tissue.

26. The use according to claim 25, wherein the diseases or conditions are selected from cancer, e.g. a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, inflammatory conditions and psoriasis.

27. The use according to claim 25 or 26, wherein the increase in phospholipase A2 activity is a least 25% compared to the normal level of activity in the tissue in question.

28. A method for selectively drug targeting to neoplastic cells, e.g., to areas within the mammalian body, preferably a human, having a phospholipase A2 activity which is at least 25% higher compared to the normal activity in said areas, by administering to the mammal in need thereof an efficient amount of the drug delivery system defined in any of the claims 16-22.

29. A liposome drug delivery system for administration of an second drug substance, wherein th second drug substance is incorporated in the liposome system, said liposome system including lipid derivatives which has (a) two aliphatic groups each of a length of at least 7, preferably at least 9, carbon atoms and (b) a hydrophilic moiety, where the lipid derivative furthermore is a substrate for endogenous phospholipase A2 (EC 3.1.1.4) to the extent that one of the aliphatic groups can be hydrolytically cleaved off, whereas the other aliphatic group remains substantially unaffected, so as to result in a fatty acid fragment and a lysolipid fragment, said lysolipid fragment not being a substrate for lysophospholipase (EC 3.1.1.5).

30. A drug delivery system according to claim 29, wherein the aliphatic group which can be hydrolytically cleaved off, is an auxiliary drug substance or an efficiency modifier for the second drug substance.

31. A drug delivery system according to claim 29 or 30, wherein the lipid derivative is a lipid derivative as defined in any of the claims 1-6.

32. A liposome drug delivery system according to any of the claims 29-31, wherein the lipid derivative constitutes a major part of the liposome system.

33. A drug delivery system according to any of the claims 29-32, wherein the second drug substance is a therapeutically and/or prophylactically active substance, in particular selected from (i) antitumor agents such as anthracyline derivatives, cisplatin, paclitaxel, 5-fluoruracil, and vincristine, (ii) antibiotics and antifungals, and (iii) antiinflammatory agents such as steroids and non-steroids.

34. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and the lipid-based drug delivery system according to any of the claims 29-33.

35. The use of a lipid-based drug delivery system according to any of the claims 29-33 as a medicament.

36. The use of a lipid-based drug delivery system according to any of the claims 29-33 for the preparation of a medicament for the treatment of diseases or conditions associated with a localised increase in phospholipase A2 activity in mammalian tissue.

37. The use according to claim 36, wherein the diseases or conditions are selected from cancer, e.g. a brain, breast, lung, colon or ovarian cancer, or a leukemia, lymphoma, sarcoma, carcinoma, inflammatory conditions and psoriasis.

38. The use according to claim 36 or 37, wherein the increase in phospholipase A2 activity is a least 25% compared to the normal level of activity in the tissue in question.

39. A method for selectively drug targeting to neoplastic cells, e.g., to areas within the mammalian body, preferably a human, having a phospholipase A2 activity which is at least 25% higher compared to the normal activity in said areas, by administering to the mammal in need thereof an efficient amount of the drug delivery system defined in any of the claims 29-33.

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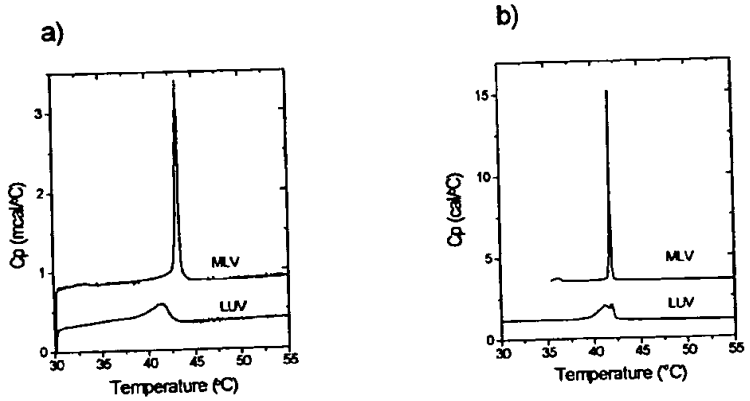

Figure 1

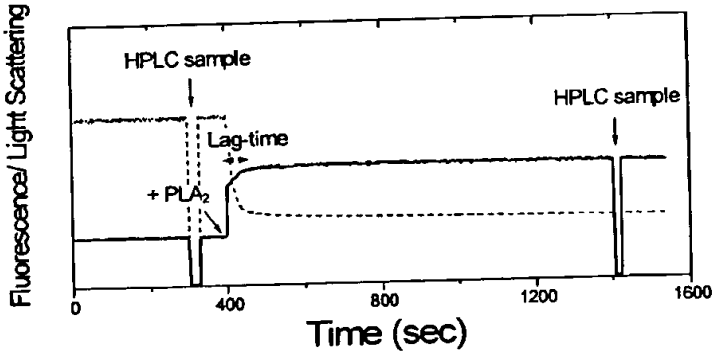

Figure 2

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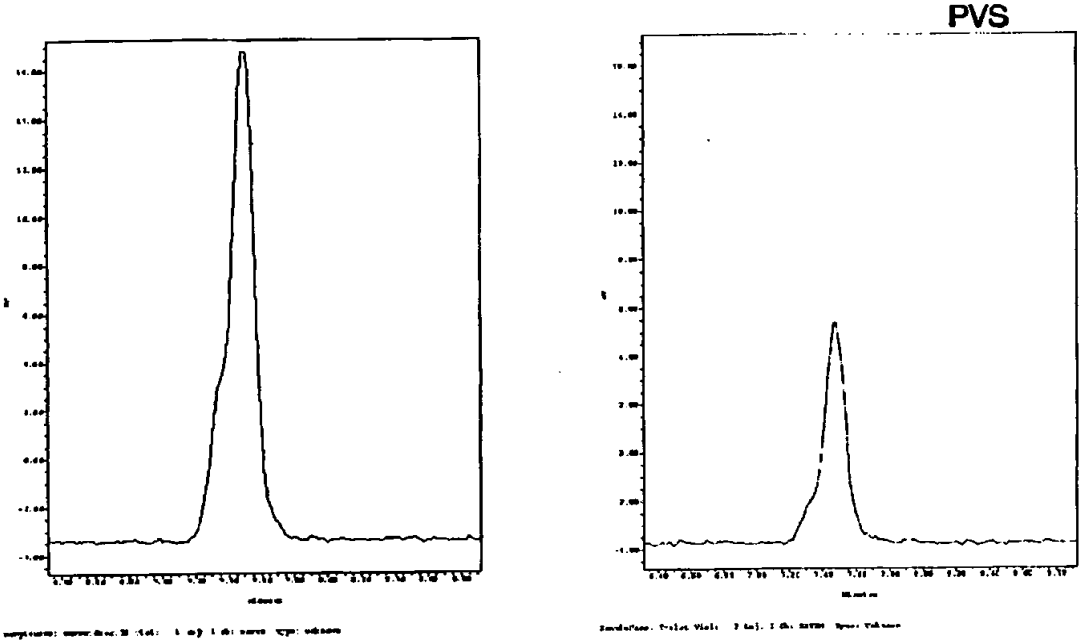

Figure 3

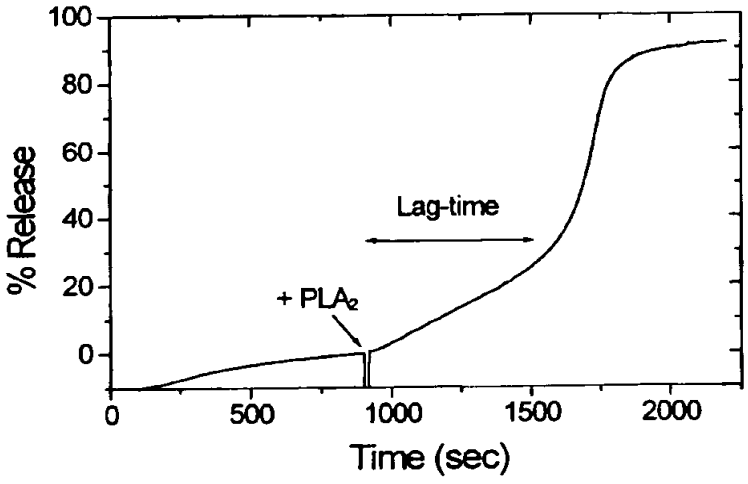

Figure 4

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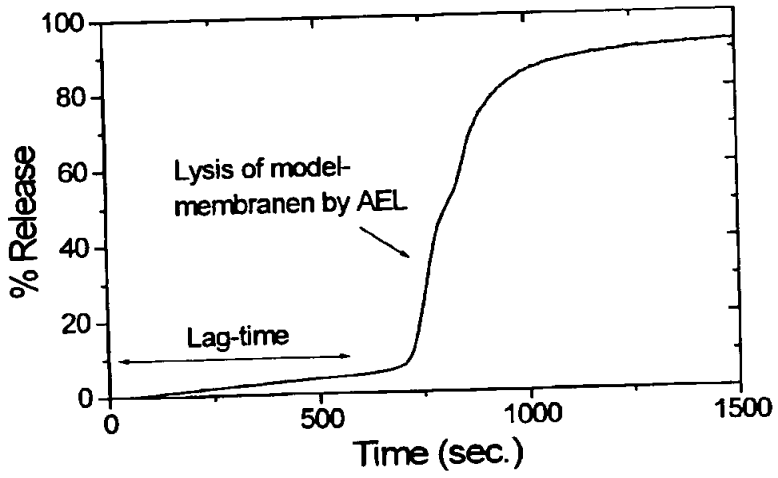


Figure 5

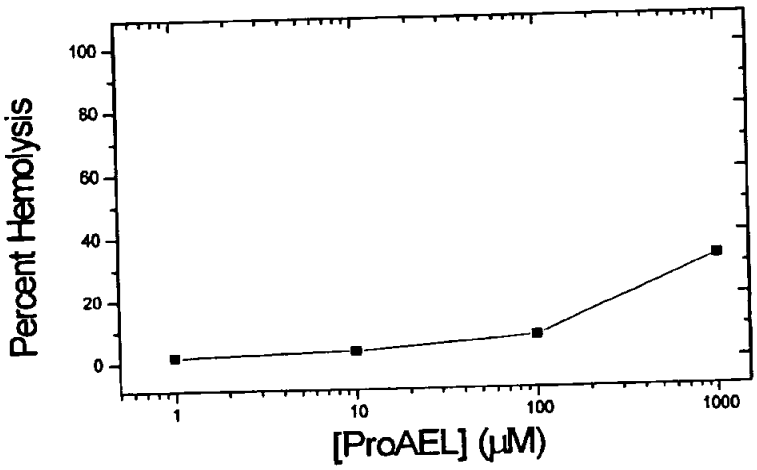


Figure 6

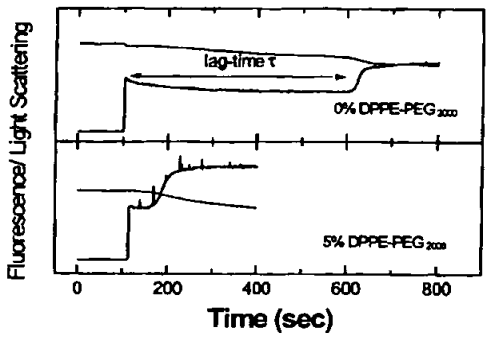
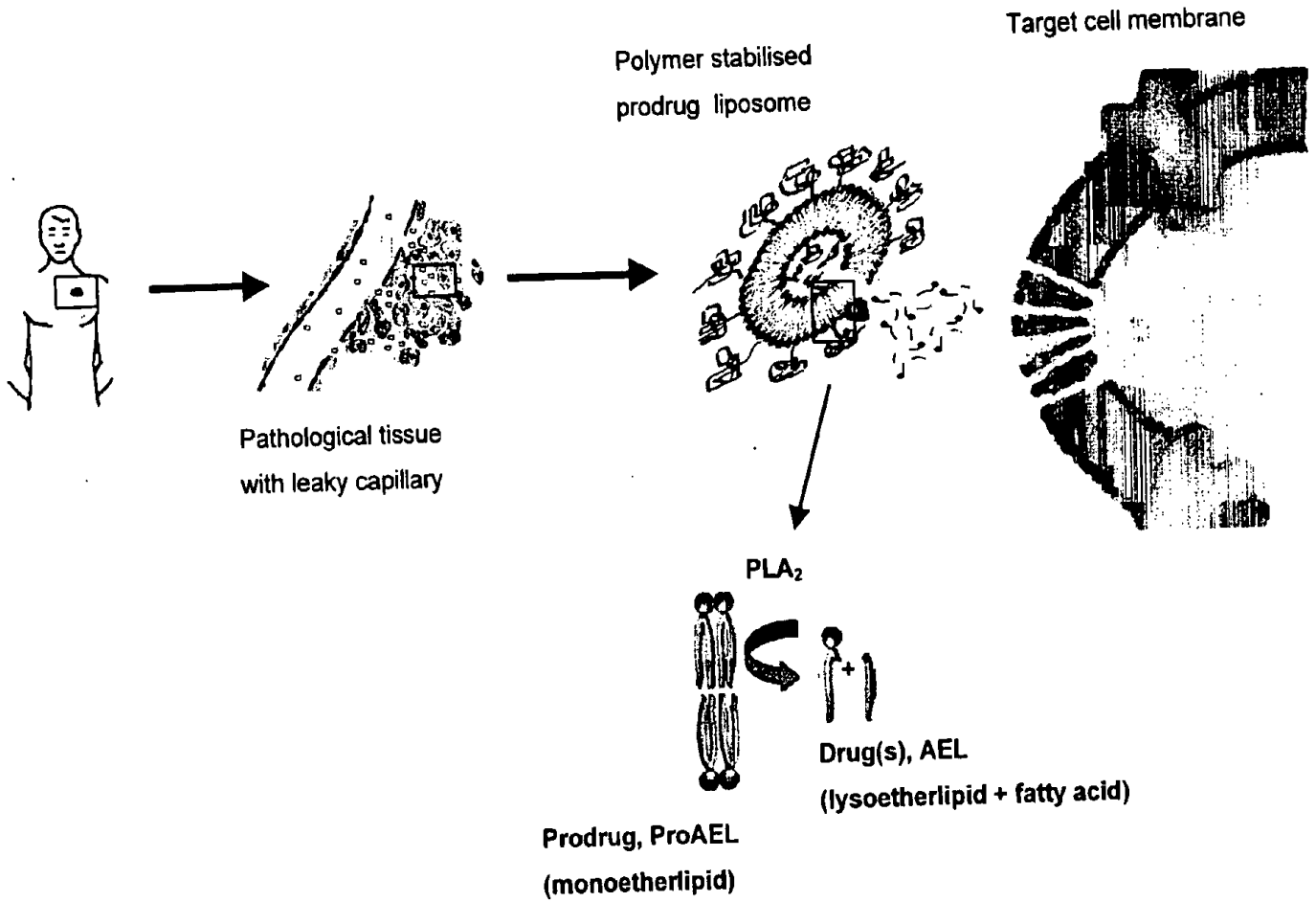


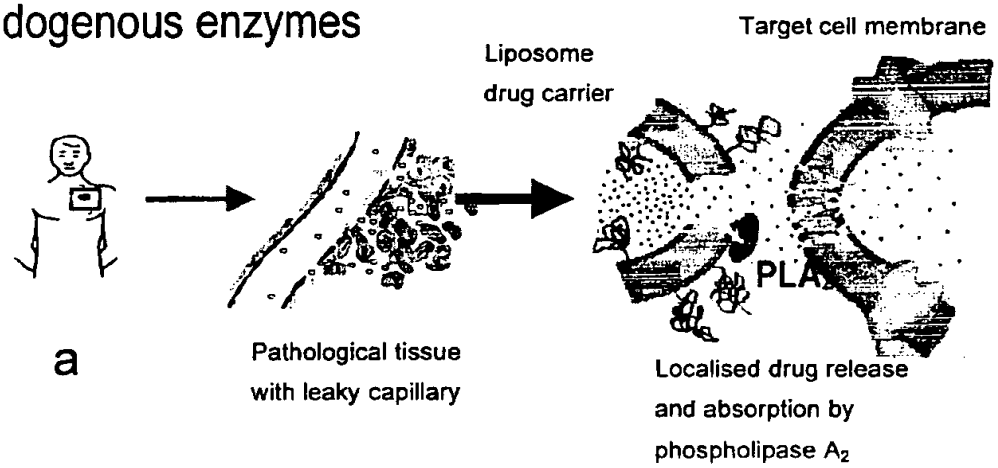
Figure 7

Figure 8

Liposomal prodrug principle: release of drug(s) by endogeneous phospholipase A_2



Liposomal drug targeting, release and absorption principle by endogenous enzymes



Biophysical drug delivery model system

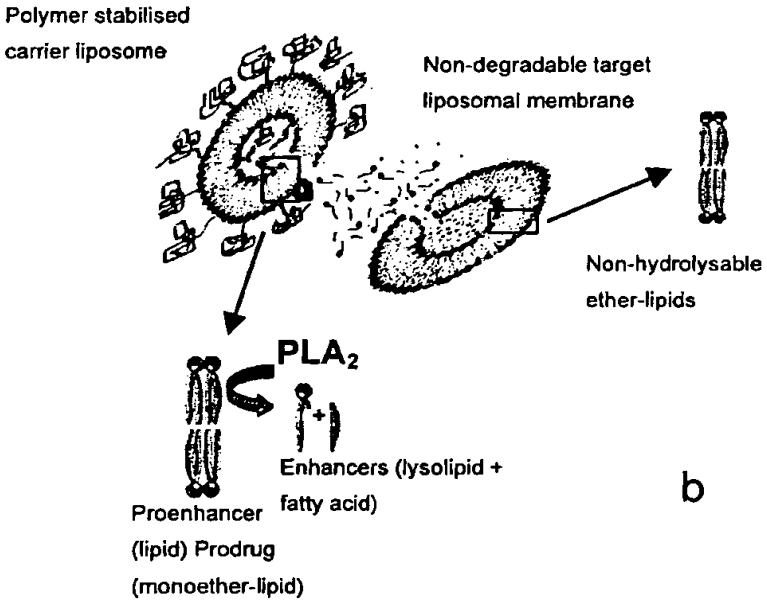


Figure 9

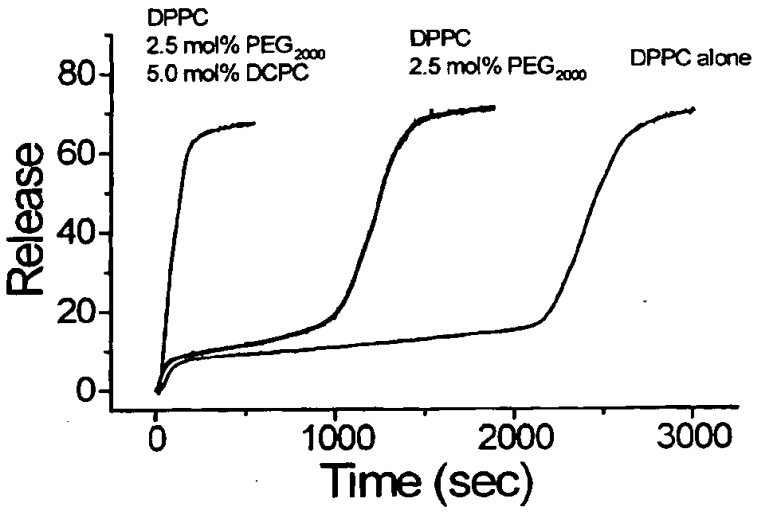


Figure 10 (a)

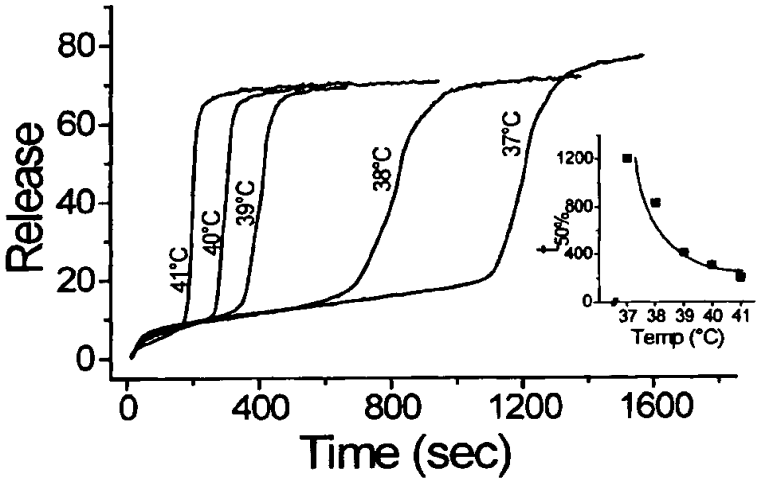


Figure 10 (b)

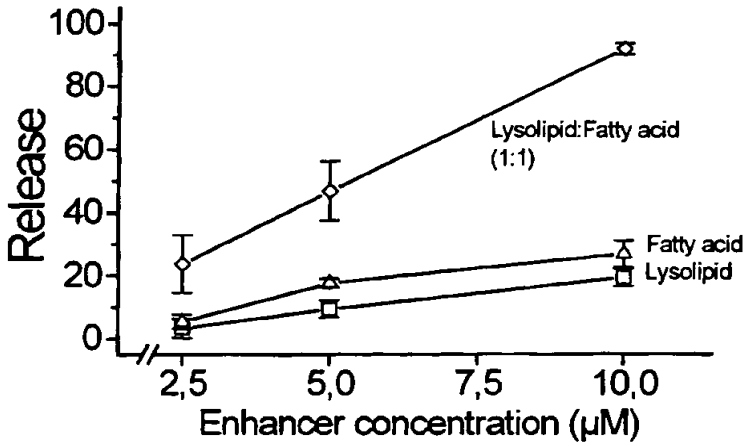


Figure 11